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Ecology of Genetically Modified Wheat

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I dedicate this thesis to
my parents

CONTENTS

GENERAL INTRODUCTION

Experimental field site at ART Reckenholz in Zurich

Potential of transgenic crops to meet the challenges of modern agriculture

Today, when the world population grows by about 83 million annually, faster than ever before in its history, modern agriculture faces unprecedented challenges to meet the increasing demand of mankind for food (Godfray *et al.* 2010). The world population has already reached 6,987 million in the middle of 2011 and is expected to further increase to over 9 billion by 2050 (Population Reference Bureau 2011). It is predicted that this will increase the world's food demand by 70–100% (Royal Society of London 2009). The means of conventional breeding and agronomic improvements have already allowed an average annual increase in world food production of 32 million tons (Alston *et al.* 2009). To meet the target of 70% increase in food production by 2050 set by FAO (FAO 2009), however, an average increase in production of at least 44 million tons per year is required. This means that a 38% increase over historical increases in food production would have to be sustained for 40 years (Tester and Langridge 2010).

Due to high population growth rates and because the most rapid population expansion usually occurs in the world's poorest countries, poverty remains one of the most serious global issues (Population Reference Bureau 2011). The Green Revolution of the 60s and 70s has lifted millions of people out of poverty through rising agricultural productivity, improving living standards and sustainable economic growth (Evenson 2003). After the Green Revolution the yields of the most important staple crops have been increasing for decades but now have reached a plateau or are even declining in some countries (Trostle 2008, Brisson *et al.* 2010, Graybosch and Peterson 2010). The Green Revolution brought to the market new highly productive but nitrogen-hungry and pesticide-demanding crop varieties. Those new crop varieties along with high-input agricultural practice and significant expansion of the areas used for growing staple crops allowed a two-fold increase in world grain production from 1960 to 2000 (Khush 2001). Further food production enhancement under the same scenario is, however, precluded by limited areas available for agricultural use and increasing soil degradation due to the intensive use of chemicals (FAO 2011).

At the same time, a rapidly urbanizing global population is demanding not only a higher food supply but also a higher quality and sustainability of agricultural production. Therefore, along with its traditional focus on increasing yields, the contemporary agricultural sector has also to face new challenges to address the environmental protection issues and rising consumer concerns for food safety and quality, and the enhancement and preservation of rural livelihoods (FAO 2011a). The

expansion of agricultural areas under the old scenario in order to increase food production would mean further destruction of forests, soil degradation and thus worsening of the environmental problems.

Biotechnology, a relatively new science, promises to help not only to increase the yields of agricultural crops but also to provide solutions to some of the environmental and social problems, for example, to expand the areas suitable for agricultural use through enhancing crop resistance to limiting environmental factors (Bohnert and Jensen 1996, Wang *et al.* 2003), to improve nutritional quality of food and food security in developing countries (Bouis 2007, Qaim 2010), and to reduce the use of chemicals in agriculture (Phipps and Park 2002). Biotechnology therefore has a potential to contribute to all the three traditional aspects of agricultural sustainability: economical, environmental and social (Serageldin 1999, Borlaug 2000, Park *et al.* 2011).

The new technology is not a panacea though and should be combined with wise assessment of the potential impacts which growing transgenic crops might have on the environment (Hails 2000, Conner *et al.* 2003).

Potential ecological risks associated with the release of transgenic crops and the problem of coexistence of conventional and GM crop production

Traditional plant breeding has already been changing plant properties for millenia. Since the time of domestication, humans have gradually modified crop varieties by selecting the best-performing individual plants. Entirely new crops were even developed by crossing the plants of different but related species (Shah and Maitra 2005). Biotechnology, however, brings plant breeding to a new level when almost any trait from any species can potentially be introduced into the crop genotype (Altman 1999).

While traditional breeding (crossing) leads to a random combination of the parental genes in a resulting offspring, genetic transformation has the advantage of a precise transfer of selected DNA sequences (Gepts 2002, Jauhar 2006). However, the site of the transgene integration is usually random and can therefore result in alteration of the inserted DNA sequence or disrupting the recipient genome and sometimes lead to undesired phenotypic effects (Filipecki and Malepszy 2006, Visarada *et al.* 2009). Improving the fitness of a plant the transgene can increase the chances for this crop to become invasive and persist outside the fields (Stewart *et al.* 1997, Warwick *et al.*

2009). On the other hand, newly introduced traits are not always advantageous for the plant and under certain environmental conditions might even impair plant performance (Bergelson and Purrington 1996, Brown 2002, Cipollini 2002). Therefore, although genetic engineering is a powerful and useful way to create additional genetic diversity for crop breeding programs, in many cases newly produced transgenic plants cannot be directly used for cultivation without preceding breeding and assessment of the potential side effects of the transgene (Snow *et al.* 2005, Andow and Zwahlen 2006, Visarada *et al.* 2009).

Among the major ecological risks which GM crops can pose to the environment are increased invasiveness of a crop, seed- and pollen-mediated gene flow to conventional varieties or to wild relatives, transgene persistence in ecosystems and potential effects on biodiversity (Altieri 2000, Pilson and Prendeville 2004, Snow *et al.* 2005, Andow and Zwahlen 2006, Sanvido *et al.* 2007).

The traits introduced by biotechnology are usually intended to improve plant performance in one or another way. Besides intended effects, such as increasing the yield or resistance to pathogens, to unfavourable environmental conditions or to herbicides, introduction of a new gene can potentially lead to unintended consequences enhancing plant ability to persist and spread within and outside agricultural fields (Wolfenbarger and Phifer 2000, Crawley *et al.* 2001, Claessen *et al.* 2005). Increased invasiveness and persistence outside agricultural fields have been reported for some herbicide-resistant GM plants, such as creeping bentgrass (Zapiola *et al.* 2008), rape (Yoshimura *et al.* 2006) and canola (Knispel *et al.* 2008). Higher fitness and potential to persist in post-cultivated areas has been also shown for transgenic Bt-rape under insect herbivore pressure (Stewart *et al.* 1997).

The GM crop itself can become invasive and persist outside agricultural areas or, as a result of outcrossing with wild relatives, the hybrids might persist and the transgene may spread in natural environments. In any case, persistence of the transgene in the environment will depend on the effect it has on fitness and life history traits of the transgenic plant (Rissler and Mellon 1996, Cummings *et al.* 2002, Claessen *et al.* 2005). The potential ecological impact of individual transgenes would thus largely depend on their phenotypic effect (Hancock 2003).

Some transgenes can have a potential to improve plant performance and reproductive success in general, under a wide range of environments. Other transgenes can enhance plant fitness under certain circumstances only, for example, in the presence

of a certain pest, under high pathogen pressure or under herbicide application. Such genes can be disadvantageous for the plant under other environmental conditions, in particular, due to the physiological costs of producing the gene product in the absence of a certain environmental factor (Bergelson and Purrington 1996). J. F. Hancock even offered grouping the transgenes by the type of impact they have on reproductive fitness of the plant: genes with neutral impact; detrimental; variable, depending on the weediness of the recipient species; variable, depending on the degree of natural biological control; or advantageous (Hancock 2003). Although sorting the transgenes according to their expected potential to improve fitness of the plant can be useful to suggest the best ways for their risk assessment, such a classification alone cannot be a basis for risk assessment without preceding testing of the GM plants under a wide set of environments. From ecological and agricultural studies it is known that genotype × environment interactions in plants can be large (Hill 1975, Schlichting 1986, Schmid 1992, Sultan 2001). It is therefore likely that the same gene can have a negative impact on plant fitness in one environment and give plants a fitness advantage in another environment. Increased fitness and persistence can occur, for example, in herbicide-resistant crops under herbicide application or in Bt transgenic crops under high pathogen pressure (Stewart *et al.* 1997, Cerdeira and Duke 2006, Culpepper 2006). Transgenic tobacco showed improved performance under drought stress but no difference in growth and yield from non-transgenic control under unstressed conditions (Pilon-Smits *et al.* 1993). Further evidence of the influence of the environmental conditions on transgene effects comes from two studies carried out on maize which found that differences in metabolic profiles and transcription observed between transgenic and control plants in the laboratory disappeared in the field (Coll *et al.* 2009, Barros *et al.* 2010).

Assessment of fitness of new transgenic plants and their performance in a wide range of agricultural and natural environments is therefore of the utmost importance when GM crop release is under consideration. It is especially relevant for the crops carrying new-generation GM traits that enhance biotic and abiotic stress tolerance and are believed to have greater potential to enhance plant fitness (Ellstrand and Hoffman 1990, Ellstrand 2001, Warwick *et al.* 2009).

Another matter of concern is that gene flow from GM plants via pollen can transfer the transgene to conventional crop varieties (Crawley *et al.* 1993, Williamson 1993, Linder and Schmitt 1994, Luby and McNicol 1995, Kwon *et al.* 2001, Warwick

et al. 2009). Pollen-mediated gene flow from GM plants to conventional varieties or wild plant species has been shown in canola, maize, bentgrass and rape (Quist and Chapela 2001, Knispel *et al.* 2008, Mallory-Smith and Zapiola 2008, Zapiola *et al.* 2008). Canola plants with several accumulated herbicide resistance genes were found persisting along highways in Canada (Knispel *et al.* 2008).

The potential of the transgenes to spread outside the agricultural areas where a GM crop is grown raises the question if the coexistence of GM and conventional farming is possible.

Defined by the European Commission as "the ability of farmers to make a practical choice between conventional, organic, and GM crop productions", co-existence concerns "the measures to achieve sufficient segregation between GM and non-GM production and the costs of such measures" (European Commission 2003). This issue was first raised by the EU Commission in 2002 and is especially relevant for organic producers committed to a worldwide consensus not to use genetically modified crops (Barth *et al.* 2002, IFOAM 2002, Binimelis 2008).

A series of authors have highlighted the difficulties to maintain coexistence between organic and GM-based agriculture (Müller 2003, Altieri 2005, Verhoog 2007, Binimelis 2008, Levidow and Boschert 2008). Some even argued that GM crops seem to be unsuitable for sustainable agriculture in the EU and therefore a moratorium on transgenic crops should be officially adopted and GMO-free regions should be declared (Müller 2003, Schermer and Hoppichler 2004, Ponti 2005, Jank *et al.* 2006).

National Research Programme 59 and Wheat Cluster — the framework for the present research project

The public attitude towards transgenic crops in Switzerland and in the European Union is overall characterised as negative: general public and farmers do not seem to be interested in growing or consuming GM crops and products (Beckmann *et al.* 2006). Most, however, agree that more research on transgenic crops and their potential risks and benefits should be done before the decision about the future use of GM plants in Switzerland can be made. The moratorium on the cultivation of genetically modified plants has been in place in Switzerland since 2005. The main reasons for the moratorium were low demand for GMOs in Switzerland and lack of scientific information about the risks and benefits of this technology.

Shortly after that, in 2007, the National Research Programme 59 (NRP59) was launched, aimed to assess the risks and benefits of deliberate release of GM crops to the environment and to provide a scientific basis for the discussion if cultivation of GM crops should be allowed or prohibited in Switzerland. The moratorium on growing GM crops in Switzerland was due to expire in November 2010. The Swiss Federal Council, however, has voted to extend it for another three years beyond this expiry date, until November 2013 (Das Schweizer Parlament 2012). The justification for the extension was to allow time for the NRP 59 to be completed and the results properly assessed.

The NRP 59 involved 29 research projects launched in 2007 and carried out by research groups from ETH Zurich, University of Zurich, federal agricultural research centres at Reckenholz-Tänikon and Changins-Wädenswil, the Research Institute of Organic Agriculture and several private companies. The projects were dealing with several species of transgenic plants, such as maize, strawberry, apple and wheat, and assessed environmental, political, social and economic aspects of GM plant cultivation.

Nine of the projects, which used transgenic wheat *Triticum aestivum* L. with introduced resistance to fungal pathogens as a model organism, formed an interdisciplinary "wheat consortium" (www.wheatcluster.ch) within the National Research Program 59. These projects shared two field sites which belonged to agricultural research stations in Pully and Zurich Reckenholz.

Transgenic wheat lines used in these studies were produced by biolistic transformation and were based on two wheat varieties: the Mexican spring wheat variety Bobwhite SH 98 26 or the Swiss variety Frisal. Bobwhite is an old wheat variety, highly susceptible to the powdery mildew pathogen *Blumeria graminis* f.sp *tritici*. The variety Bobwhite was transformed with several different alleles of *Pm3* transgenes (Brunner *et al.* 2011). *Pm3* genes, cloned from hexaploid wheat, confer race-specific resistance to the powdery mildew fungal pathogen (Yahiaoui *et al.* 2004). Another variety, used as a genetic base for transformation, was Frisal, an old Swiss variety of spring wheat which is not cultivated any longer in Switzerland. The variety Frisal was transformed with either *chitinase* or both *chitinase* and *glucanase* transgenes cloned from barley (Bieri *et al.* 2003). The expression of these transgenes should lead to an improved broad-range resistance to powdery mildew and fungal pathogens in general (Leah *et al.* 1991).

The projects within the wheat cluster focused on various aspects of transgenic plant performance and potential risks: assessed resistance of the GM wheat lines to

powdery mildew and other fungal pathogens (Brunner *et al.* 2011), gene flow to wild relative species, possible effects on non-target insect herbivore species, on soil fauna, soil bacteria and mychorrhiza (Peter *et al.* 2010, Song Wilson *et al.* 2010, von Burg *et al.* 2010, Alvarez-Alfageme *et al.* 2011, Duc *et al.* 2011). The project presented in this thesis was a part of the wheat consortium and NRP 59 and focused on the ecological behaviour of transgenic wheat plants.

Current State of Scientific Knowledge on the Ecology of GM plants

The number of studies carried out on transgenic plants is increasing along with the biotechnology development and growing public concern about the safety of GM plant cultivation (Purrington and Bergelson 1995, Dale *et al.* 2002). Most of these studies, however, are dealing with various aspects of risk assessment, assessment of agricultural yield benefits or the success of the introduced transgenes in the field trials (Altpeter *et al.* 1999, Conner *et al.* 2003). Apart from few major contributions to the field of the ecology of GM plants (Crawley 1992, Crawley *et al.* 1993, Bartsch *et al.* 1996, Marvier 2001), there were not many studies carried out by ecologists and focused on ecological features of transgenic plants, such as plant interactions with its biotic and abiotic environments, performance in agricultural or natural plant communities, competitive ability, effects on the diversity of plant communities and effects of the community diversity on GM plant performance. The number of existing studies rarely had proper genetically close controls for the comparison of GM plants with their conventional alternatives.

Possible reasons for a lack of ecological research on GM plants can be the costs of such experiments and legal difficulties to carry out research with GMOs due to the law regulations and moratoriums on GMO currently in power in many countries (Ponti 2005, Beckmann *et al.* 2006, Das Schweizer Parlament 2012). Obtaining a necessary permission can sometimes take years. In addition, many ecologists are simply not interested in working with transgenic plants and prefer to work with natural plant communities and wild species.

Transgenic plants, however, can be very promising model organisms for many ecological studies because they provide a rare opportunity to investigate the effects of single genes and traits on ecological behaviour of a plant. This is seldom possible in wild plant species or conventional crops, where particular genotypes commonly differ

in several genes from control genotypes (Somssich and Hahlbrock 1998, Strauss *et al.* 2002).

Despite a wide-spread belief among GM plant producers that the insertion of a single gene to the plant genome does not affect the overall plant phenotype, the published scientific studies point out that the transgenes can have unintended side effects on plant performance (Purrington and Bergelson 1995, Cellini *et al.* 2004, Snow *et al.* 2005, Filipecki and Malepszy 2006). In particular, there is a controversy in the scientific literature about potential changes in invasiveness of the plant after introduction of a single additional gene to the genome (Baker 1974, Williamson *et al.* 1990, Williamson 1993, Luby and McNicol 1995). Some reports suggest that if the species did not bear invasive traits before it is not very likely that the introduction of a single gene will make it invasive (Baker 1974, Luby and McNicol 1995). This, however, might not be true for the plants carrying new-generation transgenes which enhance plant resistance to biotic and abiotic stresses and pathogens and are much more likely to enhance plant fitness both in agricultural and natural plant communities and thus potentially to increase the success of its carriers in and outside crop fields (Ellstrand and Hoffman 1990, Ellstrand 2001, Warwick *et al.* 2009).

The potential of a crop to persist among other crops in the field or among natural plant communities or agricultural weeds outside the fields largely depends on the fitness of the plants and their competitive ability. Transgenic wheat has not yet been approved for cultivation and there was no previous research on the competitiveness of disease-resistant GM wheat compared to its conventional counterparts and with weeds naturally occurring in the field. In general, competition of transgenic plants with conventional varieties or with weeds has rarely been a focus of ecological research. Persistence in conventional agricultural fields or around the fields in weed communities can, however, be an important mechanism of spread and persistence for transgenic wheat. Wheat has no wild relatives in Europe (except *Aegilops cylindrica* Host.) and has low rates of natural crosspollination (Vries 1971, Guadagnuolo *et al.* 2001). The risk of crosspollination and consequent spread of fit hybrids is therefore reduced in wheat compared to some other GM crops (Hancock 2003, Gustafson *et al.* 2005). Wheat plants with introduced resistance to a common fungal pathogen affecting most wheat varieties, however, can potentially have an advantage over their conventional relatives and thus better spread by seeds and volunteer in the field after harvest or contaminate subsequent conventional crops. It is therefore crucial to obtain scientific

knowledge about the competitiveness and persistence potential of transgenic wheat at different stages of its life cycle before the release of this new GM crop to the environment.

On the other hand, resistance to pathogens, herbivores or environmental stresses is often associated with physiological costs for the plant (Bergelson and Purrington 1996, Dewitt *et al.* 1998, Brown 2002). In nature the plants mostly carry inducible resistance genes, which are regulated by the presence of the pathogen attack or stress and switch on when necessary (Heil 2001). Such resistance, called inducible, is more advantageous for the plant which does not waste resources to produce unnecessary defensive response. The transgenes of resistance, currently introduced into plants, are all regulated by constitutive promoters and thus associated with constitutive resistance (Zhu *et al.* 1994, Oldroyd and Staskawicz 1998, Bliffeld *et al.* 1999), which means they are constantly expressed, independently of the presence of the pathogen or stress. From an ecological point of view, such resistance should be costly for the plant (Bergelson and Purrington 1996). Moreover, the promoters introduced with the transgenes strongly enhance gene expression (Christensen and Quail 1996) and thus can potentially cause greater costs. A new tendency and challenge in biotechnology, transgene pyramiding, i.e. combining several transgenes in one genotype (Datta *et al.* 2002, Maruthasalam *et al.* 2007), raises the question if stacking multiple resistance genes will be advantageous for the plant or would rather incur higher physiological costs and lead to yield decrease.

An ecological alternative to transgene pyramiding could be growing several GM lines with different resistance genes in mixture. From ecological (Tilman *et al.* 1996, Hector *et al.* 1999, Roscher *et al.* 2005) and agricultural studies (Wolfe 1985, Mundt 2002) it is known that mixtures of different species or genotypes can be more productive than monocultures. Some agronomical studies have also reported that mixing the genotypes with different resistances can decrease disease spreading, prevent severe disease outbreaks and slow down adaptation of the pathogen to host defences (Wolfe 1985, Mundt 2002, Haddad *et al.* 2011). Despite reported yield benefits of the mixtures, they are not used in agriculture up to the date. The main obstacles are the costs and difficulties to separate different crops after harvest and to maintain crop purity. Transgenic plants could possibly solve the problem. Growing several lines having the same genetic background except for one gene of resistance should not cause a major problem for the end production but can potentially improve resistance of the crop and contribute to the diversity and stability of agro-ecosystems (Haddad *et al.*

2011). The lack of literature on the topic shows that this approach has not been tried before.

Gene flow via pollen from GM to conventional crops is one of the major questions in risk assessment. Answers to these questions are needed, in particular, to develop regulations about isolation distances between conventional and GM crop fields. Pollen-mediated gene flow was therefore extensively studied in many GM crops (Snow 2002, Knispel *et al.* 2008, Mallory-Smith and Zapiola 2008, Warwick *et al.* 2009). Most of the gene flow studies, however, considered only gene flow over large distances, i.e. transgene spread outside the agricultural field. Gene flow over short distances within the field is also of interest, not only for better knowledge of crossing rates of GM and non-GM plants but also to prevent possible gene multiplication in the field within one generation, reported, for example, for maize (Dietiker *et al.* 2011).

Carrying out research within the National Research Programme 59 allowed us to have a closer look at the ecology of a plant which received additional resistance genes by means of biotechnology. Availability of close genetic controls for the GM lines used in the wheat consortium provided a unique opportunity to study the effects of single genes introduced into plant genotypes on the whole phenotype of the plant and various interactions of the GM and non-GM plants with the environment.

Thesis Outline

The aim of the work presented here is to better understand the ecology of transgenic wheat and potential ecological consequences of the introduction of transgenes into common crop genotypes. Using the example of wheat genetically modified to be resistant to fungal pathogens, I considered the interactions of transgenic plants with their environment and unintended effects that the transgene might have had on plant phenotype and fitness. In a set of glasshouse and field experiments carried out over three years, I assessed the response of GM plants to abiotic environmental factors such as nutrient enrichment and fungicide application, and biotic environment, in particular their response to competition from neighboring plants, persistence in plant communities within and outside the field, their seed persistence in soil and gene flow through pollen dispersion. I was interested also in the potential effects of growing GM plants on the structure of natural plant communities in the fallow and on the behaviour of neighboring non-GM plants. In addition, we grew transgenic and conventional wheat lines under different pathogen pressure to investigate if introduced resistance genes

were physiologically costly for the plant and whether such potential costs of resistance depended on pathogen pressure. We also grew GM and non-GM wheat lines in mixtures of genotypes with different diversity levels and in monocultures to assess if mixing GM and/or conventional genotypes could be beneficial at the population level through improving pathogen resistance and reducing costs of resistance caused by the transgenes.

In *Chapter 1* I will report and discuss the results of a first field experiment where the phytometer technique was used for the first time to compare the competitive performance of GM and non-GM wheat lines. In this trial the seedlings of 15 GM and non-GM wheat lines were planted as individual plant-phytometers into plant communities composed of the same 15 lines and subject to two soil nutrient levels. The comprehensive 15 × 15 diallel design and phytometer technique allowed us to assess not only the response of the individual GM and non-GM wheat plants to competition but also the strength of the competitive environment which each wheat line as a stand provided for other individual plants.

Chapter 2 combines the results of several experiments carried out in the field and in the glasshouse in 2008–2010. In this chapter I will continue discussing the competitive interactions of GM wheat, but this time the competition with common weed species and the potential persistence of GM plants at different stages of their life cycle in weedy habitats, in fallow fields and in soil are considered. Besides the ability of the adult GM plants to withstand competition in weedy habitats and thus their potential to persist among plant communities after harvest, I also assessed the volunteering and persistence of wheat seedlings in the field and the persistence of seeds in soil. In all these experiments several abiotic factors, such as fertilizer treatment or storage conditions for the seeds, were additionally manipulated.

Chapter 3 reports the results of an assessment of four transgenic and four control wheat lines that were grown in the glasshouse under controlled conditions and in the open field in 2007–2008. In this study soil nutrient level and fungicide application were manipulated. In this experiment we investigated how the presence of a single transgene and the position of this transgene in the genome changed the plant response to several abiotic environmental factors. Besides the intended effect of the transgene, i.e. resistance to a pathogen, some unintended effects caused by the presence

of the transgene were observed. Possible reasons of such effects will be discussed in this chapter.

Chapter 4 considers the performance of three transgenic and three conventional wheat lines grown as mixtures of genotypes or as monocultures. The GM lines carried different alleles of the gene *Pm3* which confers qualitative resistance to powdery mildew pathogen. We took measurements of plant performance on individual and population level and hypothesized that a higher ratio of GM lines in the mixture and higher diversity of GM components of the mixture will reduce pathogen infection and therefore increase yields.

Chapter 5 discusses the results of the experiment which assessed the ecological relevance of costs of pathogen resistance. We investigated if GM plants resistant to powdery mildew pathogen suffer from costs of resistance in the absence of the pathogen. Individual plants of different GM wheat lines that were either based on the genetic background Bobwhite (*Pm3b* transgene) or Frisal (*chitinase* or/and *glucanase* transgene) were grown in plots sprayed with fungicide or naturally or artificially infected with powdery mildew. We grew individual plants of transgenic and control wheat lines in the field in plots sprayed with fungicide or naturally or artificially infected with powdery mildew. Furthermore, we varied the genetic diversity of the surrounding crop to study how higher diversity of the community affects the performance of individual plants with or without transgenes.

Chapter 6 reports the results of two field experiments where we tested whether rates of gene flow through pollen dispersion differed between GM and non-GM wheat lines and whether crosspollination and gene flow via pollen should be a matter of concern for transgenic wheat. The GM lines were based on two genetic backgrounds (two different wheat varieties) and contained either *Pm3b* or *chitinase*/*glucanase* transgenes. In the first experiment, outcrossing over short distances within the field was studied by planting individual plant-phytometers of one line into stands of another line. In the second experiment, outcrossing was studied over distances of 0.5–2.5 m from a central patch of pollen donors (transgenic wheat lines) to adjacent patches of pollen recipients (non-transgenic lines). Outcrossing was detected when offspring of a pollen recipient without a transgene contained this transgene in heterozygous condition.

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CHAPTER 1

Competitive Performance of Transgenic Wheat Resistant to Powdery Mildew

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Single wheat plants-phytometers planted into the machine-sown rows of a different wheat line

Abstract

Genetically modified (GM) plants offer an ideal model system to study the influence of single genes that confer constitutive resistance to pathogens on the ecological behavior of plants. We used phytometers to study competitive interactions between GM lines of spring wheat *Triticum aestivum* carrying such genes and control lines. We hypothesized reduced competitive performance of GM lines due to enhanced transgene expression under pathogen levels typically encountered in the field. The transgenes *Pm3b* from wheat (resistance against powdery mildew *Blumeria graminis*) or *chitinase* and *glucanase* genes from barley (resistance against fungi in general) were introduced with the ubiquitin promoter from maize (*Pm3b* and *chitinase* genes) or the actin promoter from rice (*glucanase* gene). Phytometers of 15 transgenic and non-transgenic wheat lines were transplanted as seedlings into plots sown with the same 15 lines as competitive environments and subject to two soil nutrient levels. *Pm3b* lines had reduced mildew incidence compared with control lines. *Chitinase* and *chitinase/glucanase* lines showed the same high resistance to mildew as their control in low-nutrient treatment and slightly lower mildew rates than the control in high-nutrient environment. *Pm3b* lines were weaker competitors than control lines. This resulted in reduced yield and seed number. The *Pm3b* line with the highest transgene expression had 53.2% lower yield than the control whereas the *Pm3b* line which segregated in resistance and had higher mildew rates showed only minor costs under competition. The line carrying both *chitinase* and *glucanase* genes also showed reduced yield and seed number under competition compared with its control. Our results suggest that single transgenes conferring constitutive resistance to pathogens can have ecological costs and can weaken plant competitiveness even in the presence of the pathogen. The magnitude of these costs seems to be related to the degree of expression of the transgenes.

Introduction

Advances in biotechnology allowed the introduction of single genes against fungal pathogens into plants (Melchers and Stuiver 2000, Gurr and Rushton 2005). The resulting transgenic plants offer a convenient model system for ecologists to study the effects of such single pathogen-resistance genes on other phenotypic traits of the transgenic plants and open up new horizons for gene × environment interaction studies (Strauss *et al*. 2002).

It is known that resistance to a pathogen might reduce plant fitness when the pathogen is absent in the environment. This is often associated with the costs resulting from the allocation of resources to unnecessary defense in a pathogen-free environment making these resources unavailable for other fitness-relevant processes (Herms and Mattson 1992, Bergelson and Purrington 1996, Heil and Baldwin 2002). Another type of costs of resistance, addressed less often, are ecological costs which arise when resistance affects the interactions between a plant and its biotic or abiotic environment in a way that reduces plant fitness (Tollrian and Harvell 1999, Heil 2002, Heil and Baldwin 2002). Ecological costs are more difficult to study because they might not be apparent under stable growing conditions indoors or on isolated plants where the range of plant × environment interactions is limited (Heil 2002). The few studies which reported ecological costs did not control for a common genetic background of resistant and susceptible plants. Moreover, those studies mostly considered induced and not constitutive resistance and thus might have been biased by side-effects of chemical treatments used for defense induction (Baldwin 1988, Heil *et al*. 2000, van Dam and Baldwin 2001).

Using genetically modified (GM) cereals as model system allowed us to control the genetic background of experimental lines and to ensure that the GM lines differed only in one resistance gene from non-GM control lines. With this we could avoid the problem that resistant plants could differ in multiple resistance and other genes from control plants which often hampers interpretation in studies of natural populations or in conventional agricultural crops (Somssich and Hahlbrock 1998, Strauss *et al*. 2002). Furthermore, promoters used with transgenes are able to enhance gene expression hundredfold and more (Rooke *et al*. 2000), thus providing a possibility to consider not only the effects of gene presence but also of strong gene expression on resistance and its potential costs.

The desired outcome of introducing genes that confer resistance into plants is that the benefits of resistance may outweigh the potential costs in the presence of the pathogen. Under disease pressure, the introduced trait should lead to better pathogen defense and thus increased fitness of the plants carrying the transgene compared with those lacking it, perhaps allowing transgenic plants or their offspring to become invasive in natural habitats (Tiedje *et al*. 1989, Ammann *et al*. 2000). Because the potential advantage will depend on the presence of the pathogen and, if ecological costs arise, on the characteristics of the environment, the competitiveness of the GM plants must be assessed against appropriate non-GM control plants under disease pressure across a range of environments (Crawley 1992, Fredshavn and Poulsen 1996). This has rarely been done in disease-resistant transgenic plants (Bartsch *et al*. 1996, Fuchs *et al*. 2004, Laughlin *et al*. 2009). Furthermore, due to the complexity of broad-range competition experiments, most studies have so far only tested a very limited number of competitive interactions.

We used a phytometer approach (Clements and Goldsmith 1924, Violle *et al*. 2009) to assess the competitiveness of six transgenic and nine non-transgenic lines and varieties (henceforth both referred to as "lines") of wheat. The phytometers of the 15 wheat lines were transplanted as seedlings into plots sown with the same 15 lines as competitive environments and subject to two different soil nutrient levels in a full mechanistic diallel setting (McGilchrist 1965, van Kleunen and Schmid 2003). Phytometers are individual plants planted into a range of environments. Originally used to measure the quality of different *environments* (Clements and Goldsmith 1924), this approach can also be applied to compare the response of different *plants* (genotypes, lines, species) to environmental conditions (Mwangi *et al*. 2007) and here allowed us to measure a wide range of plant characteristics in a large number of environments while at the same time keeping the required area for the field experiment reasonably small.

The spring wheat *Triticum aestivum* L. variety Bobwhite SH 98 26, hence abbreviated Bobwhite, transformed with the wheat *Pm3b* gene that confers resistance to powdery mildew *Blumeria graminis* f.sp. *tritici* (DC.) Speer (Yahiaoui *et al*. 2004), and variety Frisal with introduced fungal resistance genes *chitinase* and *glucanase* from barley (Leah *et al*. 1991) were used to study the effects of single pathogen-resistance genes on the competitive ability of GM plants. Since the same lines were used as phytometers and competitive environments (full 15×15 mechanistic diallel), it was possible to assess the effect of every line as a competitive environment on the average

performance of every line planted as a phytometer into this environment and to estimate mildew infection and competitiveness of individual phytometers of every line surrounded by plants of the other lines (competitive environment).

Apart from providing information about the effects on plant × environment interactions of single pathogen-resistance genes, the assessment of the competitiveness of transgenic and conventional wheat in crop environments contributes to understanding the potential risks associated with offspring of GM plants potentially occurring and competing with conventional wheat in subsequently sown fields. Furthermore, a potentially enhanced performance of phytometers when grown with another line ("away environment") instead of its own ("home environment") would suggest a positive effect of growing wheat in line mixtures, an effect abundantly found in biodiversity experiments (Balvanera *et al*. 2006) and, for example, caused by decreased disease levels at stand level in mixtures (Smithson and Lenne 1996, Zhu *et al*. 2000, Zeller *et al*. 2012).

The work presented here is part of a joint project of several research groups called "wheat consortium" within the framework of the Swiss National Research Program 59 "Benefits and risks of the deliberate release of genetically modified plants" (www.NRP59.ch). A set of the other research experiments within NRP59 studied agronomic properties of the wheat lines in common agricultural trials, gene × abiotic environment interactions in the glasshouse and in the field (Zeller *et al*. 2010), disease resistance and gene expression (Brunner *et al*. 2011) and the impact of the GM lines on other organisms (von Burg *et al*. 2010, Alvarez-Alfageme *et al*. 2011, von Burg *et al*. 2011).

Here we asked the following questions: (1) Do the introduced transgenes improve resistance to mildew and do they affect the performance of the phytometers grown under competition (main effects of transgenes)? (2) How do the nutrient and the competitive environments affect resistance to mildew and phytometer competitive performance (main effects of environments)? (3) Do the differences between transgenic and control lines vary across nutrient and competitive environments (overall transgene × environment interactions)? (4) Do transgenic and control lines behave differently if planted into their own rather than into different lines as competitive environments (home vs. away contrast of transgene × environment interactions)? We found that transgenic constitutive resistance to a fungal pathogen can affect plant × environment

interactions and reduce the competitiveness of the GM plants with strong transgene overexpression.

Materials and Methods

Plant material

We used four transgenic lines derived from the Mexican spring wheat variety Bobwhite and two transgenic lines derived from the Swiss variety Frisal in our experiment. Spring wheat *T. aestivum* is a predominantly self-pollinating species with hexaploid genome and growing season from early spring to late summer (in Switzerland). Bobwhite and Frisal were chosen because these varieties are known for high transformation efficiency and regeneration frequency (Pellegrineschi *et al*. 2002, Bieri *et al*. 2003). Furthermore, they are both susceptible to powdery mildew, yet to different degrees (Bobwhite > Frisal).

The transgenic lines of Bobwhite (*Pm3b*#1–4) and their non-transgenic control sister lines (Sb#1–4) were produced by biolistic transformation in four different transformation events. *Pm3b*#1–3 lines carried a single copy of the transgene *Pm3b*, and *Pm3b*#4 line carried one full-length and one non-functional truncated copy (Brunner *et al*. 2011). Their non-transgenic sister lines were null-segregants that had undergone the same tissue culture processes and thus had acquired the same potential somaclonal variation as their respective transgenic sisters. Southern blot and PCR analysis showed that Bobwhite and the null-segregants did not carry endogenous copies of the *Pm3* gene (Brunner *et al*. 2011). The *Pm3b* gene confers race-specific resistance to powdery mildew and was cloned from the hexaploid wheat landrace Chul (Yahiaoui *et al*. 2004). The seeds used in this study were obtained from homozygous GM and control lines that had passed through five generations of sexual reproduction by self-pollination.

The performance in monoculture and the transgene expression of the lines *Pm3b*#1–4 have been described by two companion studies (Zeller *et al*. 2010, Brunner *et al*. 2011). The constitutive ubiquitin promoter from *Zea mays* L. ensured that the transgene was expressed at a high level: *Pm3b* transcript levels were 11, 55 and 5 times higher in *Pm3b*#1, *Pm3b*#2 and *Pm3b*#3, respectively, compared to the donor landrace Chul according to the results of the field assessment of the three *Pm3b* lines and the landrace Chul in 2009 (at that time line *Pm3b*#4 was not available for comparison) (Brunner *et al*. 2011). The expression levels of the *Pm3b* gene quantified in the leaf

samples from the field in 2008 by a reverse transcription, quantitative real-time polymerase chain reaction (RT-qPCR) were similar among *Pm3b*#1, *Pm3b*#3 and *Pm3b*#4 lines, while the line *Pm3b*#2 showed around five times higher expression levels (Brunner *et al*. 2011). Partial gene silencing and consequent segregation in resistance were observed in the *Pm3b*#3 line, where part of the plants showed high resistance and another part was susceptible to mildew (see Figure 2 in Brunner *et al*. 2011 for details). In monoculture, some unintended effects such as chlorotic leaves and partial male sterility were observed in line *Pm3b*#2 and in a highly resistant subset of *Pm3b*#3. We hypothesized that these unintended effects were related to a very high transgene expression (Brunner *et al*. 2011).

The GM lines derived from the variety Frisal carried either a barley seed *chitinase* gene (line A9 *Chi*) or both a *chitinase* and a *β-1,3-glucanase* gene (line A13 *Chi/Glu*) (Bieri *et al*. 2003). Chitinases and glucanases are known for their anti-fungal effect. The expression of these pathogenesis-related genes should result in increased quantitative resistance to mildew (Leah *et al*. 1991, Zhu *et al*. 1994). The seeds used for the field experiment were obtained from the sixth generation of transgenic lines A13 *Chi/Glu* and A9 *Chi*. No transgene silencing occurred in these lines (C. Diaz Quijano *et al.*, unpublished data). In the absence of sister lines that had undergone the same tissue culture as the transgenic Frisal lines, we used ordinary non-transgenic Frisal plants as the control line. Here we present the results of the first field experiment carried out with these plants. All GM lines used were produced as model plants for the National Research Program 59 and were not intended for agricultural commercialization.

In addition to the 11 lines already mentioned, four further wheat lines were used: ordinary non-transgenic Bobwhite plants that had not passed through tissue culture and the three commercial non-transgenic Swiss varieties: Casana, Fiorina and Toronit. The latter were used as reference "out-groups" to compare differences caused by the transgenes *within* varieties with differences *between* varieties, and thus to verify whether the characteristics of the GM plants fall within the range of natural variation between conventional varieties of wheat—the test of equivalence required by the European Food Safety Authority for risk assessment of GM plants (EFSA 2011).

Field experiment

The field experiment took place in 2008 at a research station in Zurich-Reckenholz, Switzerland. The 15 wheat lines were sown in 60 plots of 7×1.08 m each, in a

randomized complete block design with four replicate blocks. Each plot represented one of the 15 wheat competitive environments for the phytometers. The two edge subplots of 1×1.08 m in each plot were used for a split-plot treatment, i.e. fertilizer application vs. control. Fertilizer was applied twice: when the plants had reached phenological stage 11 on the so-called "Zadoks" scale (Zadoks *et al*. 1974) and again when they had reached stage 39, to one of the two subplots in each plot (two times 3 g N m^{-2} as "Ammonsalpeter 27.5", Lonza, Visp, Switzerland). The natural field soil provided plants with phosphorous, potassium and magnesium (80, 235 and 234 mg kg^{-1}, respectively).

In each 1×1.08 m subplot, 400 wheat seeds were sown in six rows with a distance of 18 cm between the rows using an Oyjord plot drill system (Wintersteiger AG, Ried, Austria). Five seedlings per subplot were randomly chosen and marked shortly after germination for later assessment of mildew incidence in the sown competitive environments. All plots were sprayed with the herbicide cocktail Concert SX (40% Thifensulfurone, 4% Metusulfurone-methyl; Stähler Suisse AG, Zofingen, Switzerland) and Starane super (120 g L^{-1} Bromoxynil, 120 g L^{-1} Ioxynil, 100 g L^{-1} Fluroxypyrmetilheptilester; Omya Agro AG, Safenwil, Switzerland) at the beginning of May. Mildew infection occurred naturally. As a subset of these plots (environments but not the phytometers) had provided the plant material for one of our previous publications (Zeller *et al*. 2010), it was possible to compare the results of the present phytometer study with the results of the assessments of the plants sown in the plots as competitive environments, at least in those cases where the same lines (*Pm3b* and sister lines) and traits were assessed.

Phytometers

In February 2008, 3600 individual seeds of the 15 wheat lines (the same lines as used in the field plots) were germinated in a climate-controlled glasshouse (day/night temperature: 21/16 C°; additional light: 14 h/10 h day/night period, daily watering by hand) at the Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Switzerland. When the seedlings reached phenological stage 11–12 on the Zadoks scale (Zadoks *et al*. 1974) the temperature in the glasshouse was lowered to 5 C° to slow down the growth. In March 2008, when the plants in the field reached the same phenological stage and similar size as the plants in the glasshouse, the seedlings were transplanted from the glasshouse to the field plots and inserted into the test

environments described in the previous section. These seedlings, grown under standard conditions in the glasshouse, were used as phytometers to assess their phenotypic response to competitive environments and fertilizer application. In our experiment, the phytometers did not differ in their performance (plant height, number of leaves, phenological stage) among the wheat lines at the stage of transplanting and during early stages (phenological stage 14−15) of growth in the field. This indicates that, if the transplanting influenced the growth of the seedlings, all the phytometers responded to it similarly.

Thirty phytometer seedlings representing the 15 wheat lines were introduced into each 1×1.08 m subplot. Before the phytometers were planted, already established seedlings of the competitive environment were removed from the rows to free space for five phytometers per row (six rows per subplot; Figure S1 in Supplemental Material). Thus, the ratio phytometers:competitors was 30:370 in the subplot. The distance between neighboring phytometer plants in a row was 20 cm. As a result, phytometers of each of the 15 lines occurred in each of the 15 lines as competitive environments. This corresponds to a full mechanistic diallel design (McGilchrist 1965, van Kleunen and Schmid 2003). Each phytometer line was represented twice in each subplot.

Measurements

We recorded plant height and phenological stage (Zadoks *et al*. 1974) of all phytometer plants 53 days after planting. Plant height was measured from the soil level to the highest point of the plant. The incidence of powdery mildew infection was assessed for phytometers and also for marked plants of the sown competitive environment 80 days after planting when infection reached its maximum. It was measured as a presence/absence of the disease symptoms on individual plants, and then a percentage of plants infected with the pathogen out of all the plants was calculated for each wheat line. After ripening, all phytometers were cut at ground level and separated into vegetative and reproductive parts (spikes). All plant material was dried at 80 C° (vegetative parts) and 25 C° (reproductive parts) and weighed. We counted the spike number per phytometer plant, threshed the reproductive parts, determined the seed number per plant and obtained the total mass of seeds per plant. In the following, the total mass of seeds and the seed number per plant are called yield and seed number, respectively. The phytometer data were used to characterize the competitiveness of different wheat lines. For each fertilizer treatment and each trait, we calculated the

relative performance values for each phytometer line by dividing the subplot means of the line through the mean value this phytometer line reached in its own competitive environment (McGilchrist 1965, Allard and Adams 1969, McGraw 1985). This was used as a test for home vs. away effects, corresponding to a main-diagonal contrast within the transgene × environment interaction term (Joshi *et al*. 2001).

Data analysis

Data were analyzed with classical mixed-model analysis of variance (ANOVA) using the statistical software GenStat (VSN International Ldt. 2010). The treatment model consisted of the factorially crossed phytometer lines and competitive environments (mechanistic diallel) and fertilizer application. The error model consisted of phytometer plants nested within subplots, subplots nested within plots and plots nested within blocks. The terms of the treatment model were tested against the appropriate terms of the error model: competitive environment varied among plots, fertilizer application among subplots and phytometer line within subplots (Figure S2 in Supplemental Material). Residual plots were examined to identify outliers and to check if the assumptions of normality and homoscedasticity were fulfilled. Three hierarchical models were used for the analysis (Figure S2): (1) comparing the groups of GM lines with the groups of control lines (i.e. 4 *Pm3b* lines vs. 4 Sb lines, A9 *Chi* and A13 *Chi/Glu* vs. Frisal), (2) and (3) comparing GM and control lines pairwise (i.e. *Pm3b*#1 vs. Sb#1, A9 *Chi* vs. Frisal, A13 *Chi/Glu* vs. Frisal, etc.). All data were log-transformed to fulfill ANOVA assumptions of normality and homoscedasticity. The binary mildew incidence data were analyzed using multiple logistic regression with mixed-model analysis of deviance (McCullagh and Nelder 1989).

First we analyzed the originally measured variables to identify the differences in phytometer performance and the effects of competitive environment and fertilizer application. Then we analyzed the relative performance values (see previous section) to compare the competitive ability of phytometer lines independently of their different performance in "pure stands" (i.e. phytometer plant in its own competitive environment). For this we calculated the log-ratio away/home as a dependent variable for the analysis (see e.g. Petermann *et al.* 2008). For each phytometer line × fertilizer × competitive environment combination and for each trait measured, there were four replicate log-ratios according to the four blocks in the field. However, the relative

performance means (in percentage, back-transformed from the log-scale) are presented in text and figures.

In June 2008, 1093 out of 3600 phytometer plants were damaged by vandals. These plants were excluded from the analysis of the traits measured after the damage had happened. ANOVA showed that the damage by vandalism occurred randomly across the phytometer lines and did not interfere with the effects of the factors of interest.

Results

Mildew Incidence

Main effects of transgenes (phytometer lines)

Powdery mildew incidence reached its maximum in the field 80 days after transplanting of the phytometers. Phytometers carrying the *Pm3b* gene showed the desired decrease in mildew incidence (*Pm3b* lines vs. Sb lines phytometer contrast: $P<0.001$); this decrease was up to five-fold compared with control lines (Figure 1). The difference between *Pm3b* lines and Sb lines explained 12.4% of the total variation in mildew incidence and exceeded the variation among the three conventional wheat varieties Toronit, Casana and Fiorina 41.3 times (Table S1 in Supplemental Material).

Each *Pm3b* line had significantly lower mildew incidence than its corresponding Sb line (all pairwise phytometer comparisons of *Pm3b* and Sb lines: $P<0.001$). The four *Pm3b* lines, however, differed significantly from one another in mildew incidence (4 *Pm3b* lines phytometer contrast: $P=0.01$). Overall, line *Pm3b*#2 had the lowest and line *Pm3b*#3 had the highest mildew scores with, respectively, 6% and 14% of the phytometers infected. The four control Sb lines only marginally differed from each other in mildew incidence (4 Sb lines phytometer contrast: $P=0.059$) and were highly susceptible to the pathogen (up to 62% of the plants infected).

The results obtained with phytometers were similar to the results of other mildew assessments of the same *Pm3b* and Sb wheat lines done in the field at subplot level (Zeller *et al*. 2010, Brunner *et al*. 2011): the *Pm3b* lines showed higher resistance to the pathogen than the sister lines, *Pm3b*#2 line being the most resistant to powdery mildew among the four GM lines.

The lines derived from the susceptible Mexican variety Bobwhite had a 14-fold increased mildew incidence compared with the lines derived from the Swiss wheat variety Frisal (Bobwhite vs. Frisal phytometer contrast: $P<0.001$).

The lines A9 *Chi* and A13 *Chi/Glu* showed very low mildew incidence (1.5 and 0.7%, respectively), however, mildew incidence was also low in the Frisal control line. Mildew incidence in Frisal plants never exceeded 7% of all phytometers in any line x fertilizer treatment combination (Figure 1). In the fertilized subplots, where the mildew infection was generally higher than in the unfertilized subplots (see next paragraph), the control Frisal line did have higher mildew incidence than the two GM-lines of Frisal (interaction Fertilizer × A9 *Chi* and A13 *Chi/Glu* vs. Frisal: P=0.002; A9 *Chi* and A13 *Chi/Glu* vs. Frisal phytometer contrast: P=0.047 in fertilized environments).

Main effects of environments (soil nutrients and wheat competitive environments)

Application of fertilizer led to a twofold increase in mildew incidence of phytometers (main fertilizer effect: P<0.001). The competitive environment also affected mildew incidence (main competitive environment effect: P=0.001). Higher mildew rates were observed for the phytometers introduced into mildew-susceptible wheat environments (Figure 1), Sb lines representing the most "infective" environments, in which average mildew incidence among the phytometers reached 29.9%. Mildew occurred 3.6 times more often in phytometers grown in mildew-susceptible Sb environments than in those grown in *Pm3b* plots (*Pm3b* vs. Sb lines competitive environment contrast: P<0.001). Mildew incidence did not differ between the phytometers grown in Frisal transgenic and control competitive environments.

Overall transgene × environment interactions

The difference in mildew incidence between *Pm3b* and Sb lines increased 1.7-fold with nutrient addition (interaction Fertilizer × *Pm3b* lines vs. Sb lines: P<0.001; Frisal results mentioned above). Competitive environment also affected the magnitude of the difference in mildew incidence between *Pm3b* and Sb lines (interaction Competitive environment × *Pm3b* lines vs. Sb lines: P=0.024). This difference was 3.4 times stronger in mildew-susceptible Sb than in mildew-resistant *Pm3b* competitive environments.

Phytometer Performance

Main effects of transgenes (phytometer lines)

Being planted into competitive environments, transgenic *Pm3b* lines on average developed 45.4% less seeds, 39.4% lower yield and 4.8% lower vegetative mass, and

had a more advanced phenological stage and plant height (4.9% and 4.3%, respectively) than control Sb lines (*Pm3b* lines vs. Sb lines phytometer contrasts: P<0.001 for all traits; Figure 2A). In addition, the four *Pm3b* lines differed among each other in performance (4 *Pm3b* lines phytometer contrast: P<0.001 for seed number, yield, spike number, vegetative mass and plant height, P=0.023 for phenological stage); the four control Sb lines in contrast differed only in vegetative mass (4 Sb lines phytometer contrast: P=0.018) with lower values observed for the lines Sb#2 and Sb#3 than for the other two lines (Figure 2A; pairwise comparisons in Tables S2 and S3).

The GM line *Pm3b*#2, known for the highest transgene expression (Brunner *et al*. 2011), had the lowest performance among the four *Pm3b* GM lines: yield was reduced by 53.2%, seed number by 48.1% and vegetative mass by 27.3% compared with the corresponding control Sb#2 (*Pm3b*#2 vs. Sb#2 phytometer contrast: P<0.001 for yield, seed number and vegetative mass). This line also had 8.8% more advanced phenological stage and 13.2% taller plants than the control line (P<0.001 for phenological stage and plant height).

The three other *Pm3b* lines differed less from their controls. The line *Pm3b*#1 had 12.7% reduced yield, 17.5% reduced seed number and 10.2% reduced vegetative mass compared with its sister line Sb#1 (*Pm3b*#1 vs. Sb#1 phytometer contrast: P=0.047, P=0.005 and P=0.015, respectively). *Pm3b*#4 showed 7.9% lower yield and 18.4% lower seed number (*Pm3b*#4 vs. Sb#4 phytometer contrast: P<0.001 for yield and seed number) and had slightly advanced phenological stage compared with its sister line Sb#4 (P=0.008 for phenological stage). Line *Pm3b*#3, which had higher mildew incidence than the three other *Pm3b* lines (Figure 1), differed significantly from its sister line Sb#3 only in seed number (*Pm3b*#3 vs. Sb#3 phytometer contrast: P=0.001 for seed number).

The variation explained by the difference between *Pm3b* and Sb lines exceeded the variation among the three conventional wheat varieties Casana, Toronit and Fiorina for the traits yield, seed number and vegetative mass several times (Tables S2 and S3 in Supplemental Material).

The lines derived from the Swiss wheat variety Frisal had an advanced phenological stage and a 1.5-fold increased plant height compared with the lines derived from the Mexican variety Bobwhite. Bobwhite lines, on average, had a 1.3-fold increased seed number per plant and yield, 1.2-fold increased spike number and 1.5-

fold increased vegetative mass compared with Frisal lines (Bobwhite vs. Frisal phytometer contrast: P<0.001 for all the traits).

Line A9 *Chi* carrying the transgene for chitinase production did not differ in its performance from the Frisal control line, whereas line A13 *Chi/Glu*, carrying two transgenes for chitinase and glucanase, had a 1.2-fold decreased yield and 1.3-fold reduced seed number compared with the Frisal control line (A13 *Chi* vs. Frisal phytometer contrast: P=0.017 for yield, P<0.001 for seed number).

Main effects of environments (soil nutrients and wheat competitive environments)

Nutrient addition enhanced plant growth and development (main fertilizer effect: P<0.001 for all the traits) causing a 2.4-fold increase in yield, 2.3-fold increase in seed number and vegetative mass, 1.4-fold increase in spike number, an advance in phenological stage and a 1.2-fold increase in plant height of the phytometers.

Competitive environment had a strong influence on phytometer growth. Phytometers grown in transgenic *Pm3b* competitive environments had a 1.4-fold yield, 1.5-fold seed number, 1.2-fold spike number and 1.3-fold vegetative mass compared with phytometers grown in Sb competitive environments (*Pm3b* vs. Sb lines competitive environment contrast: P<0.001 for vegetative mass, yield and spike number, P=0.001 for seed number). For these traits, the differences between *Pm3b* and Sb competitive environments exceeded the variation among the three conventional-wheat-variety environments (Tables S2 and S3), mirroring the results obtained when analyzing main effects of phytometers (see previous section).

Phytometers which had Frisal lines as competitive environments had delayed phenological development compared with those planted into Bobwhite lines as competitive environments (Bobwhite vs. Frisal competitive environment contrast: P=0.004). The phytometers planted in the different Frisal environments (A9 *Chi*, A13 *Chi/Glu* lines and mother variety) only varied in phenological stage, which was delayed in phytometers grown in the transgenic A9 *Chi* compared with those grown in Frisal environment (A9 *Chi* vs. Frisal competitive environment contrast: P=0.045).

Overall transgene × environment interactions

Nutrient addition did not significantly change the magnitude of the differences in performance between *Pm3b* and control Sb lines. However, the plants of the line A9 *Chi* were 4% shorter than those of the Frisal control line in fertilized subplots and did

not differ from them in unfertilized subplots (interaction Fertilizer × A9 *Chi* vs. Frisal: P=0.02 for plant height).

The competitive environments Frisal vs. Bobwhite significantly affected the differences in yield, seed number and vegetative mass between *Pm3b* lines and control Sb lines (interaction *Pm3b* lines vs. Sb lines phytometer contrast × Bobwhite vs. Frisal competitive environment contrast: P=0.001 for yield, P=0.002 for seed number, P=0.034 for vegetative mass). The difference between *Pm3b* and Sb phytometers in yield increased 2.1-fold, in seed number 1.7-fold and in vegetative mass 5-fold when the plants were grown in Frisal as compared to Bobwhite environments.

Home vs. away contrast of transgene × environment interactions (relative performance)

Overall, yield, seed number and vegetative mass were higher (see term Overall mean in Tables S4 and S5: P=0.009, P=0.032 and P<0.001 for log-ratios of yield, seed number and vegetative mass) and plant height and phenological stage were lower in "away" than in "home" environments (P=0.027 for log-ratio of plant height, P=0.001 for log-ratio of phenological stage). This was indicative of higher performance (biomass) due to reduced light competition (lower height) of phytometers in "away" environments.

On average, Bobwhite control lines and conventional Swiss varieties performed better in away than in home environments whereas the opposite was the case for GM lines (Figure 2B): *Pm3b* lines had 52.5% lower relative yield, 44% lower relative seed number, 25.4% lower relative spike number and 32.5% lower relative vegetative mass than the Sb control lines (*Pm3b* lines vs. Sb lines phytometer contrast: P<0.001 for log-ratios of yield, seed number, spike number and vegetative mass). However, on average, relative plant height and phenological stage were 4% higher for the *Pm3b* lines than for the Sb lines (P=0.005 and P=0.012, respectively).

Not all lines contributed to the same degree to the mentioned average differences between GM and non-GM lines. The four *Pm3b* lines differed significantly in their relative yield, spike number, seed number, plant height and vegetative mass (4 *Pm3b* lines phytometer contrast: P<0.001; Tables S4 and S5). Lines *Pm3b*#2 and *Pm3b*#4 had the most negative log-ratios for these traits, indicating their weaker performance in competition with the other wheat lines than in "home" environments. In particular, *Pm3b*#2 line had 60% reduced yield, 56.5% reduced seed number, 22.6% reduced spike number and 50% reduced vegetative mass in "away" compared with

"home" environments. *Pm3b*#4 line showed 36.5% reduction in yield, 38.3% reduction in seed number, 34.4% reduction in spike number and 29.9% reduced vegetative mass in "away" compared with "home" environments. The performance of the other two *Pm3b* lines in "away" environments was similar to that in their own environment. In particular, line *Pm3b*#3 differed from the Sb#3 control line only by having higher relative phenological stage and plant height (*Pm3b*#3 vs. Sb#3 phytometer contrast: P=0.009 and P<0.001 for log-ratios of phenological stage and plant height, respectively). This confirms the results on absolute performance of *Pm3b*#3 line under competition: this line had only minor differences compared with its sister control line.

Frisal transgenic lines A9 *Chi* and A13 *Chi/Glu* had slightly advanced phenological development and plant height in away compared to home environments, whereas the Frisal control line was more phenologically advanced and taller in home than in away environments (A9 *Chi* and A13 *Chi/Clu* vs. Frisal phytometer contrast: P<0.001 for log-ratios of plant height and phenological stage). The line A13 *Chi/Glu* and Frisal variety had increased vegetative mass in away as compared to home environments, whereas A9 *Chi* line showed no such effect (A9 *Chi* vs. A13 *Chi/Clu* phytometer contrast: P=0.026; A9 *Chi* vs. Frisal phytometer contrast: P=0.025 for log-ratio of vegetative mass).

Nutrient addition reduced the overall positive away/home log-ratios of yield, seed number, vegetative mass and phenological stage (main fertilizer effect: P=0.014, P=0.001, P=0.007, P=0.021 for log-ratios of yield, seed number, vegetative mass and phenological stage, respectively), indicating that line mixtures may be less beneficial under high than under low soil nutrient conditions.

Discussion

Main effects of transgenes

Our first question was whether the introduced transgenes improved plant resistance to powdery mildew and whether this resistance incurred any costs for GM plant fitness when the plants were grown under competition and pathogen levels typically encountered in the field. Resistance to mildew was substantially increased in GM lines carrying the *pm3b* transgene, as expected, but generally not in GM lines carrying the *chitinase* and *glucanase* transgenes, presumably because the latter were introduced into the old Swiss wheat variety Frisal which already had an elevated level of resistance to

the pathogen. The difference in mildew incidence between the GM lines and the control line of Frisal could only be observed in fertilized environments where plants were more susceptible to the pathogen. It is conceivable, therefore, that under higher pathogen pressures the difference between Frisal GM lines and the Frisal control line in pathogen resistance would also have become (more) visible.

Increased mildew resistance, however, did not lead to enhanced growth and competitive performance of the tested GM lines in the presence of the pathogen. On the contrary, the plants with *pm3b*-mediated resistance to mildew had on average lower yield and reduced seed number than their corresponding control lines. This suggests that the costs were high enough to overcome the benefits of being resistant to the pathogen, reducing the plants' fitness and their ability to withstand competition from neighbors. Similar effects, i.e. lower relative fitness under competition, have been previously reported for the plants with chemically induced resistance to pathogens (Heil *et al*. 2000, van Dam and Baldwin 2001). Analysis of uninfected seedlings (Yahiaoui *et al*. 2004, Brunner *et al*. 2011) had previously shown that the GM lines of Bobwhite expressed the *Pm3b* gene constitutively and five- to several-hundred-fold more strongly than did the wheat landrace Chul from which the *Pm3b* gene was taken (Brunner *et al.* 2011). Although only two *Pm* genes have been cloned in wheat (Yahiaoui *et al.* 2004, Cao *et al.* 2011) and no detailed time-course expression data for indigenous *Pm* genes have been published to date, the expression analysis in resistant wheat landrace Chul (S. Brunner *et al*., unpublished data) indicate that the *Pm3b* gene is also constitutively expressed with its indigenous promoter. The control Mexican wheat variety Bobwhite and the null-segregants used as sister control lines carried no indigenous *Pm* genes. Therefore we suggest that the differences in performance between the *Pm3b* and the control lines could be explained by the high expression of the *Pm3b* gene in transgenic lines. However, because we could not compare the performance of our transgenic Bobwhite lines with that of the landrace Chul, we cannot exclude the possibility that even with the original promoter the *pm3b* might have reduced plant performance under the prevailing pathogen pressure.

These costs of resistance also indicate that, at least under the environmental conditions encountered in our field experiment, the mildew-resistant GM lines do not have a higher chance than conventional lines to establish and persist as volunteers in wheat habitats. There is a discussion in the literature if the addition of a single gene can cause a crop to become weedy (Baker 1974, Williamson *et al*. 1990, Luby and McNicol

1995). Some authors state that weediness arises from many different characters and, therefore, if the species previously had no weedy characteristics, the addition of one or a few genes should not alter its competitiveness to such a large extent as seen in our study (Baker 1974, Luby and McNicol 1995). Our results support the other point of view that even small genetic changes such as the insertion of a single gene in a new genetic background can cause large ecological alterations affecting genotype × environment interactions (Williamson *et al*. 1990, Williamson 1994, Dale *et al*. 2002). This would apply even though in our case the effects of the transgene were in the direction of decreased rather than increased potential weediness in the presence of the pathogen.

In accordance with the different transformation events leading to the four *Pm3b* lines with different expression levels, we found significant differences between the four transgenic lines in their performance and interactions with the different competitive environments. Thus, line *Pm3b*#2, which showed the highest resistance to powdery mildew, was the weakest competitor, re-enforcing the view that transgene-caused, high mildew resistance was negatively correlated with plant performance. When the average yields are plotted against the average mildew incidence for all Frisal and Bobwhite lines (Figure 3) it can be seen that the relationship is positive at low infection levels for GM lines of Bobwhite and negative at high infection levels for control lines of Bobwhite (in fertilized subplots). This suggests that at very high levels of plant defense there is no gain for a plant to become even more resistant, rather increased resistance in this case could lead to a reduction in performance.

All *Pm3b* lines had enhanced transgene expression compared with the normal expression in the wheat landrace from which the *Pm3b* gene originated (see Zeller *et al*. 2010, Brunner *et al.* 2011 for details). The line *Pm3b*#2, however, showed fivefold higher expression than the average of lines *Pm3b*#1, *Pm3b*#3 and *Pm3b*#4 (Brunner *et al*. 2011). This indicates that the overexpression of the gene that confers resistance could be a cause of the changes in the plants' interactions with their environment. Because the corresponding control lines passed through the same transformation procedure as *Pm3b* lines but did not show reduced competitive performance, we assume that the reduced performance in *Pm3b* lines was a consequence of the physiological costs they paid for the increased resistance to the pathogen (Bergelson and Purrington 1996, Brown 2002, Heil and Baldwin 2002). Another GM line, *Pm3b*#3, had only minor or no performance differences compared with its control line.

According to the gene expression and segregation analysis data (Brunner *et al*. 2011), this line showed transgene silencing of different intensity in a large proportion of the plants and segregation in resistance (about 44% susceptible plants in the sixth generation). It also showed higher average mildew incidence than the other three GM lines in our phytometer experiment (Figure 1). The gene silencing could be an explanation for the lower costs of resistance found in *Pm3b*#3 line.

The data obtained at individual plant level in the phytometer experiment supported the results of our previous glasshouse and plot-level field assessments of the same *Pm3b* and Sb lines grown from seed (Zeller *et al*. 2010, Brunner *et al*. 2011). The transplanted phytometers of *Pm3b*#2 line showed the same altered phenotypes as did the sown plants (Zeller *et al*. 2010, Brunner *et al*. 2011). These alterations, strong resistance to mildew and weaker performance of the *Pm3b*#2 line under competition most likely were a consequence of the transgene overexpression and not due to events occurring during tissue culture because the sister plants of control line Sb#2 had undergone the same tissue culture events. In a previous study (Zeller *et al*. 2010), where we assessed the ecological behavior of sown plants of the different lines of Bobwhite at subplot level, we found that the transgenic lines *Pm3b*#1–4, compared with their sister lines, also had increased levels of Ergot infection, suggesting that further, non-observed pleiotropic effects might have influenced the yield of GM plants (including phytometers) in our study.

Line A13 *Chi/Glu*, which possessed both *chitinase* and *glucanase* transgenes, had lower yield and seed number than the Frisal control line. In accordance with this observation, line A13 *Chi/Glu* also showed an increased resistance compared with the control line in fertilized subplots. Again it appears that additional investment into pathogen resistance, which was already elevated in the Frisal control line, was costly for the Frisal GM line containing two transgenes. That performance was not reduced in the Frisal line with only one transgene (A9 *Chi*) suggests that the degree of defense matters for the costs of defense. We conclude that a high constitutive level of mildew resistance has negative effects on the performance of GM wheat plants and thus reduces their potential to persist in conventional agricultural fields. It could be suggested that lower levels of intrinsic resistance to pathogens might produce better-performing GM plants. From a risk perspective, however, such plants would have to be evaluated again in a range of biotic and abiotic environments in similar experiments as the one

presented here to test their or their offsprings' potential to successfully compete with non-GM plants.

Main effects of environments

Our second question was how variation in the abiotic (fertilization) and biotic environment (competition with other wheat lines) may influence resistance to mildew and the performance of phytometer plants. Nutrient addition enhanced powdery mildew incidence in both transgenic and conventional wheat lines. Similar effects were reported in previous studies with non-transgenic plants, where the severity of mildew infection was shown to be related to the nitrogen supply of the host (Last 1953, Bainbridge 1974, Shaner and Finney 1977, Chen *et al*. 2007). Lines with high mildew incidence proved to be infective environments as shown by the higher mildew incidence of phytometers in these (see Figure 1). This is a well-known epidemiological effect (Wolfe 1985) and relevant when considering planting mixed-line crops because in the same way as more susceptible neighbors can increase infection in less susceptible target plants so can more resistant neighbors reduce infection in less resistant target plants. In a further field experiment we found that indeed overall mildew incidence in line mixtures was lower than in the average single-line stand (Zeller *at al.* 2012), an observation previously made in a genetic diversity experiment with the wild plant species *Solidago canadensis* (Schmid 1994).

Fertilization enhanced plant growth and reproduction in all the investigated wheat lines. In addition, the performance of the phytometer plants was strongly influenced by the type of competitive environment. Phytometers planted with transgenic *Pm3b* lines as competitors outperformed those planted into competitive environments of Sb lines. This is in accordance with the results of the analysis of the main effects of transgenes (see previous section). Phytometers which had Frisal variety as a competitive environment generally had weaker performance than those in Bobwhite environments. The congruence between phytometer-line and competitive environment-line effects, i.e. high-performing phytometer lines also providing highly competitive environments thus in turn reducing phytometer performance, indicates that phytometers do provide realistic measures of competitive ability.

Overall transgene × environment interactions

The third question asked whether transgenic wheat lines responded to variations in nutrient and competitive environments in the same way as did conventional lines. The difference in mildew incidence between GM lines and control increased with the addition of nutrients. A similar effect has been previously described in non-transgenic plants, where the increased severity of infection due to fertilization was more pronounced in susceptible than in resistant crop varieties and therefore the magnitude of the difference between these varieties increased with nutrient addition (Shaner and Finney 1977). In accordance with these observations, the difference in mildew incidence between the transgenic lines and control lines also became stronger in mildew-susceptible than in mildew-resistant competitive environments.

Significant transgene × competitive environment interactions were observed for the majority of fitness-related traits and reflected more sensitive responses to competition for transgenic *Pm3b* lines of variety Bobwhite than for other lines. Our findings indicate that a single gene that confers constitutive resistance to a pathogen might strongly affect genotype × environment interactions if expressed at high level, making ecological costs of resistance apparent even in the presence of the pathogen. The fact that the differences between GM and control lines in pathogen level and plant performance vary depending on the environment points to the importance of testing transgenic plants under a set of biotic and abiotic environments in realistic field conditions (Crawley 1992, Fredshavn and Poulsen 1996). The phytometer approach (Clements and Goldsmith 1924, Violle *et al*. 2009) could be a useful tool for this kind of studies.

Using this approach we could assess competitive interactions and the response to fertilizer treatments in 15 different transgenic and conventional wheat lines simultaneously on a relatively small area of less than 130 m^2 in the field. An advantage of the approach is the possibility to incorporate several biotic and abiotic factors that might affect the performance and competitive ability of test plants simultaneously into a single and comprehensive experimental setting (Clements and Goldsmith 1924, Mwangi *et al*. 2007, Violle *et al*. 2009). In addition to measuring the competitiveness of the individual phytometer plants, the experimental design also allowed us to assess the competitive strength of the environment provided by each wheat line. Where the phytometers benefited from being in a certain competitive environment it indicated that the line representing this environment was not a strong competitor. Furthermore, the

overall effect that phytometers performed better in the neighborhood of plants from other lines (away) rather than their own line (home) suggests that line mixtures should perform better than the average line monoculture at plot level (see next section); a positive biodiversity effect normally tested with large setups of plots varying in diversity level (Balvanera *et al*. 2006). In the future the phytometer approach could be used in field studies of transgenic plants to facilitate the identification of promising new breeds and increase the flexibility and power of ecological risk assessment.

Relative performance in home vs. away environments

The fourth question was whether transgenic and non-transgenic lines behave differently if planted into their own (home) rather than into different lines as competitive environments (away). Most of the phytometer plants benefited if their neighbors belonged to a different line (mixture effect). This is consistent with findings in biodiversity experiments (Balvanera *et al*. 2006). The transgenic *Pm3b* lines of variety Bobwhite, however, showed lower relative values (performance in "away" as compared to "home" environments) than the corresponding control lines for four out of six fitness-related traits. Only the line with partial gene silencing, *Pm3b*#3, showed no such costs under competition with the other lines. Because the GM line *Pm3b*#2 suffered most in line mixtures, it appears that this line paid a particularly high fitness costs for its elevated mildew resistance under competition. This supports the recent findings that competition might increase the magnitude of the costs of resistance (Agrawal 2000, Heil *et al*. 2000, van Dam and Baldwin 2001). Our results, however, also point to the importance of the type of the competitor and the expression level of the resistance gene. The resistant line with the highest transgene expression, *Pm3b*#2, appeared to be especially sensitive to inter-line competition, whereas the differences between this line and its sister control line became smaller when the competitor was represented by its own genotype. Interestingly, the reduced performance and fertility under competition with the other wheat lines (relative performance) was also observed in the *Pm3b*#4 line (Figure 2B) which is known to carry an additional non-functional truncated copy of the transgene. As the level of the gene expression did not differ strongly between the lines *Pm3b*#1 and *Pm3b*#4, it could be speculated that the impaired competitiveness of this line was caused by position effects via the disruption of endogenous genes (Rooke *et al*. 2000, Brunner *et al*. 2011).

Transgenic plants with unintended phenotypes, including low fecundity, often arise during molecular plant breeding (Snow *et al*. 2005, Filipecki and Malepszy 2006). They are usually detected early and their ecological performance is not further investigated (Cellini *et al*. 2004). In our case, however, the transgenic lines had higher performance than their non-transgenic control lines in the glasshouse under high pathogen pressure and only in the field this fitness advantage reversed (Zeller *et al*. 2010). Although there have been several studies that measured the costs of resistance in transgenic plants or in plants with induced defenses (Baldwin 1988, Bergelson and Purrington 1996, Agrawal 2000, Baldwin and Hamilton 2000, Heil *et al*. 2000, van Dam and Baldwin 2001, Heil 2002, Heil and Baldwin 2002, Strauss *et al*. 2002, Chen *et al*. 2006), only few of those have considered the effects of intra- and interline competition on the costs of resistance (Baldwin and Hamilton 2000, van Dam and Baldwin 2001, Chen *et al*. 2006). One of these studies found that the benefits of transgenic resistance to herbivores in rice disappeared when the plants were grown in competition with other genotypes instead of a pure stand (Chen *et al*. 2006).

Our results confirm this precedence and demonstrate that a transgene increasing plant resistance to a pathogen and constitutively expressed at a high level may reduce rather than increase a plant's competitive ability and thus lower its probability to persist outside its own field. An early study of Crawley *et al.* showed that herbicide-tolerant transgenic lines of rape showed no evidence to be more successful or more invasive than their conventional counterparts in the *absence* of herbicide treatment and even showed weaker invasive potential in some aspects, such as in seed survival on burial (Crawley *et al*. 1993). In our experiment, however, the costs for plant fitness and competitiveness could be observed even in the *presence* of the pathogen against which the GM lines had increased resistance. Very likely we would have observed even higher costs in our study if the pathogen would have been excluded in our field trial.

Apart from these findings, nutrient addition negatively affected the ability of plants to coexist in the mixtures. This supports the theory that fertilization increases competition between genotypes or species for scarce resources and in particular light (Wilson and Tilman 1993, Hautier *et al*. 2009). It would therefore be even more difficult for competitively weak transgenic plants to persist in well fertilized agricultural habitats.

Conclusions

In conclusion, this study shows that a single gene conferring resistance against a particular fungal pathogen can have large and negative effects on plant performance under realistic field conditions even if these conditions include the presence of the pathogen. We interpret these large costs in resistant plants as a consequence of altered gene regulation, in particular enhanced gene expression level, which was here achieved with a strong promoter introduced with the gene that confers resistance. This indicates that altered regulation in a single gene may strongly affect plant fitness and the way the plant interacts with the environment, in particular changing a plant's competitive ability.

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Figures

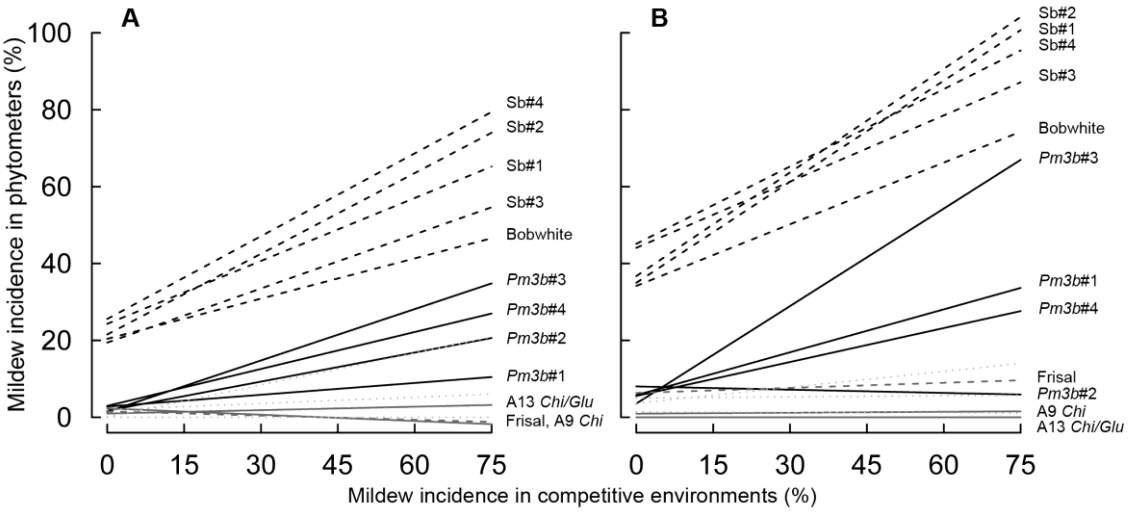


Figure 1. Mildew incidence in phytometers of 15 wheat lines grown with the same lines as competitive environments. Left chart (A): soil with low nutrient level. Right column (B): soil with high nutrient level (fertilized subplots). The mildew incidence in phytometers is plotted as a function of the mildew incidence of the competitive environments (linear regression lines), demonstrating differences among phytometer lines and increased infection in phytometer plants in pathogen-susceptible environments. Mildew incidence is the percentage of plants infected with the pathogen. Solid black lines: lines *Pm3b*#1–4; dashed black lines: lines Sb#1–4 and Bobwhite; solid grey lines: transgenic A9 *Chi* and A13 *Chi/Glu* lines; dashed grey lines: Frisal control line; dotted grey lines: three Swiss conventional wheat varieties (Casana, Toronit and Fiorina).

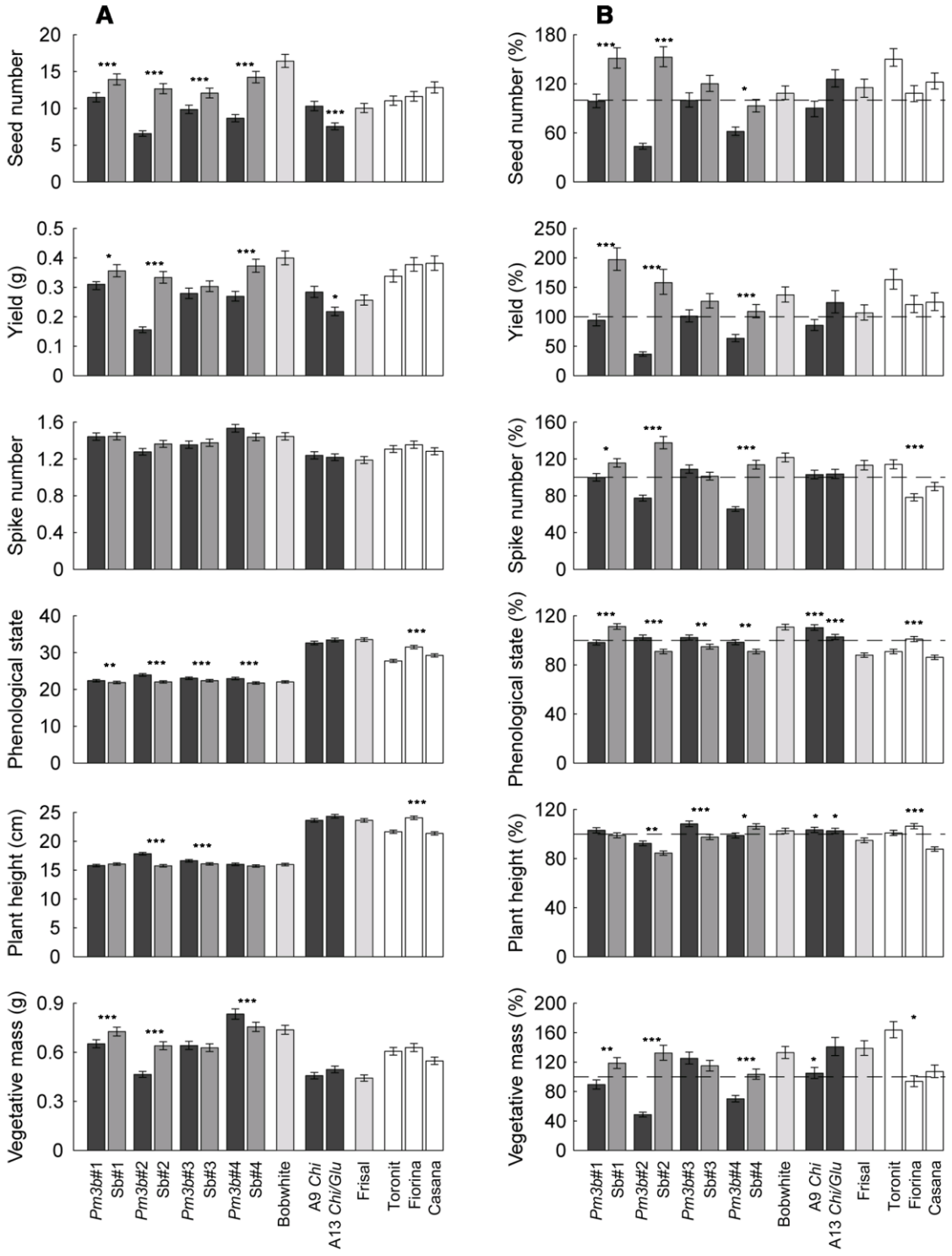


Figure 2. Performance of the 15 wheat lines grown with the same lines as competitive environments. Left column (A): average performance of the transgenic and conventional lines across 15 competitive environments. Right column (B): relative performance of the investigated wheat lines under competition with other lines expressed as a percentage of the estimates in their own environment. The data for high

and low nutrient treatments are pooled. Dashed lines denote 100% (i.e. log-ratio = 0: same performance in own and foreign competitive environment). Bars represent means ± standard errors back-transformed from log scale. Five grades of the grey scale indicate groups of wheat lines; from dark to light: transgenic lines, the genetically closest control (sister lines), wheat varieties used for transgene insertion and modern conventional wheat varieties. The significant differences between the *Pm3b* and corresponding control Sb lines, between Frisal and A9 *Chi* line, Frisal and A13 *Chi/Glu* line and among the three conventional varieties Fiorina, Casana and Toronit are shown with asterisks: *** – $P<0.001$, ** – $P<0.01$, * – $P<0.05$.

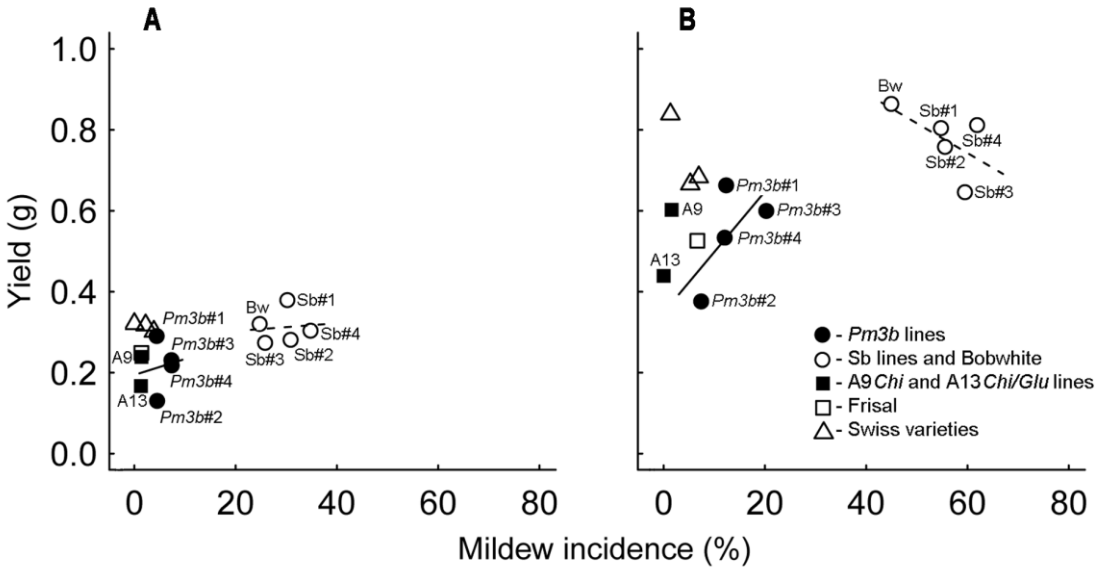


Figure 3. The relationship between mildew incidence and yield in 15 wheat lines. Left chart (A): soil with low nutrient level. Right column (B): soil with high nutrient level (fertilized subplots). The solid and dotted lines are linear regression lines for the groups of means for transgenic *Pm3b* lines and for control Sb lines and variety Bobwhite. Mildew incidence is a percentage of plants infected with the pathogen. The data for 15 wheat competitive environments are pooled.

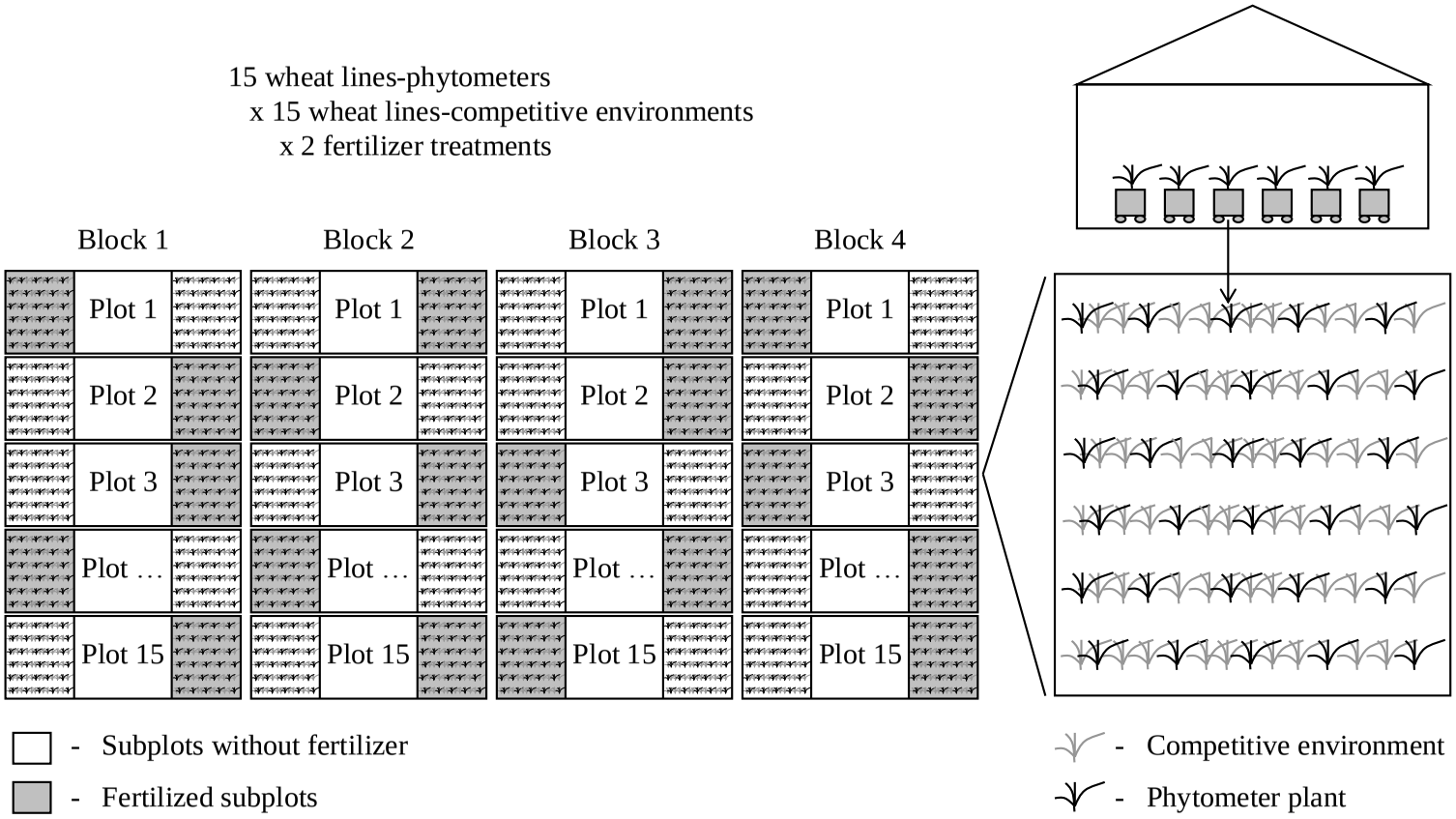
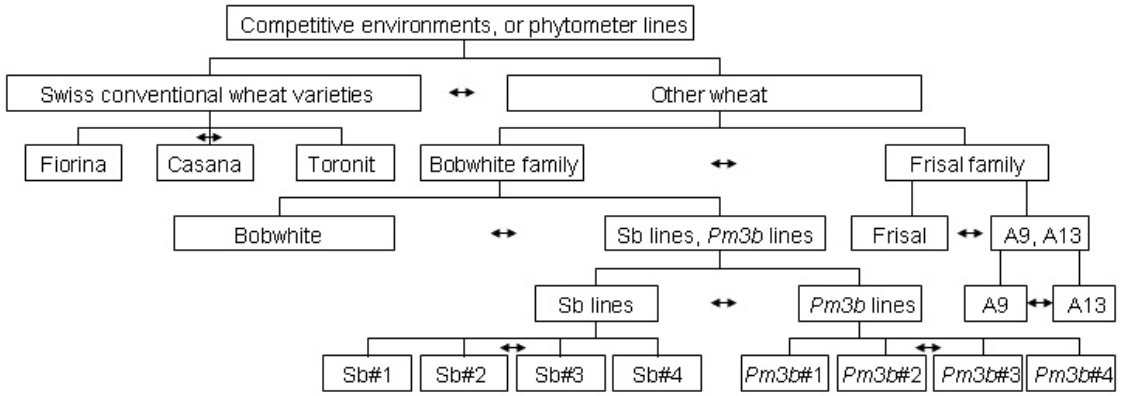


Figure S1. Design of the phytometer experiment.

Version 1.



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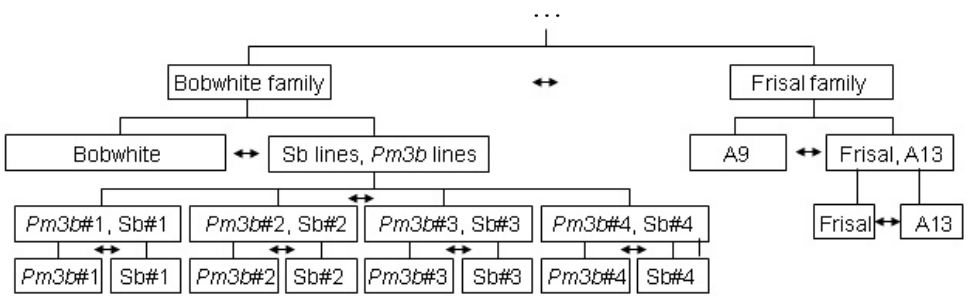

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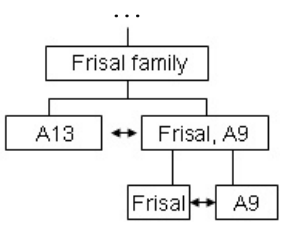

Figure S2. The structure of orthogonal contrasts used in the extended ANOVA models.

Table S1. Analysis of deviance table showing the effects of fertilizer, competitive environment, differences between GM and non-GM lines and their interactions on mildew incidence

Simple model

Source of variation	Mildew incidence		
	df	%SS	F pr.
Block	3	1.1	0.018
Competitive environment (Comp.env.)	14	5.2	0.001
Plot	42	4.3	0.040
Fertilizer	1	3.1	<.001
Comp.env.×Fertilizer	14	1.2	0.202
Subplot	45	2.7	0.949
Phytometer lines	14	26.8	<.001
Comp.env.×Phytometer lines	196	7.1	0.067
Plot×Phytometer lines	593	18.2	<.001
Phytometer lines×Fertilizer	14	1.2	<.001
Residual	1513	29.0	
Total	2449	100	

Extended model

Source of variation	Mildew incidence		
	df	%SS	F pr.
Block	3	1.1	0.018
Competitive environment (Comp.env.)	14	5.2	0.001
Plot	42	4.3	0.040
Fertilizer	1	3.1	<.001
Comp.env.×Fertilizer	14	1.2	0.202
Subplot	45	2.7	<.001
Phytometer contrasts (Phytometer lines effect):			
Swiss vs. other wheat	1	5.7	<.001
3 conventional Swiss varieties	2	0.3	<.001
Bobwhite vs. Frisal	1	7.6	<.001
Bobwhite vs. Sb lines	1	0.3	<.001
Pm3b lines vs. Sb lines	1	12.4	<.001
4 Sb lines	3	0.1	0.059
4 *Pm3b* lines	3	0.3	0.001
A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.1	0.008
A9 *Chi* vs. A13 *Chi/Glu*	1	0.0	0.331
Pairwise comparisons:			
Pm3b#1 vs. Sb#1	1	3.1	<.001
Pm3b#2 vs. Sb#2	1	3.7	<.001
Pm3b#3 vs. Sb#3	1	1.7	<.001
Pm3b#4 vs. Sb#4	1	4.1	<.001
A9 *Chi* vs. Frisal	1	0.1	0.007
A13 *Chi/Glu* vs. Frisal	1	0.1	0.006
Comp.env.×Swiss vs. other wheat	14	0.4	0.069
Comp.env.×3 conventional Swiss varieties	28	0.7	0.671
Comp.env.×Bobwhite vs. Frisal	14	0.7	0.088
Comp.env.×Bobwhite vs. Sb lines	14	0.3	0.728
Comp.env.× *Pm3b* lines vs. Sb lines	14	0.8	0.024
Comp.env.×4 Sb lines	42	2.0	0.014
Comp.env.×4 *Pm3b* lines	42	1.6	0.138
Comp.env.×A9 *Chi* and A13 *Chi/Glu* vs. Frisal	14	0.3	0.756
Comp.env.×A9 *Chi* vs. A13 *Chi/Glu*	14	0.2	0.947
Plot×Phytometer lines	593	18.2	<.001
Fertilizer×Swiss vs. other wheat	1	0.1	0.119
Fertilizer×3 conventional Swiss varieties	2	0.0	0.487
Fertilizer×Bobwhite vs. Frisal	1	0.0	0.758
Fertilizer×Bobwhite vs. Sb lines	1	0.0	0.843
Fertilizer×*Pm3b* lines vs. Sb lines	1	0.2	<.001

Table S1 continues

Source of variation	Mildew incidence		
	df	%SS	F pr.
Fertilizer×4 Sb lines	3	0.3	0.002
Fertilizer×4 *Pm3b* lines	3	0.1	0.225
Fertilizer×A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.2	0.002
Fertilizer×A9 *Chi* vs. A13 *Chi/Glu*	1	0.4	<.001
Residual	1513	29.0	
Total	2449	100	

Table S2. ANOVA table showing the effects of fertilizer, competitive environment, differences between GM and non-GM lines and their interactions on three yield characteristics

Simple model

Source of variation	Yield (log)			Spike number (log)			Seed number (log)		
	df	%SS	F pr.	df	%SS	F pr.	df	%SS	F pr.
Block	3	4.2	<.001	3	2.9	<.001	3	3.5	<.001
Competitive environment	14	8.3	<.001	14	5.9	<.001	14	8.5	<.001
Plot	42	6.4	<.001	42	3.4	0.376	42	5.5	<.001
Fertilizer	1	17.2	<.001	1	9.7	<.001	1	17.0	<.001
Comp.env.×Fertilizer	14	0.5	0.596	14	0.8	0.709	14	0.4	0.696
Subplot	45	1.9	0.007	45	3.3	<.001	45	1.8	0.017
Phytometer lines	14	5.9	<.001	14	2.0	<.001	14	6.7	<.001
Comp.env.×Phytometer lines	196	5.4	0.071	196	6.1	0.583	196	5.7	0.058
Plot×Phytometer lines	593	13.9	0.888	598	19.2	0.171	593	14.3	0.818
Phytometer lines×Fertilizer	14	0.2	0.896	14	0.7	0.085	14	0.2	0.811
Residual	1406	36.0		1522	45.9		1406	36.2	
Total	2342	100		2463	100		2342	100	

Extended model

Source of variation	Yield (log)			Spike number (log)			Seed number (log)		
	df	%SS	F pr.	df	%SS	F pr.	df	%SS	F pr.
Block	3	4.2	<.001	3	2.9	<.001	3	3.5	<.001
Competitive environment contrasts:									
Swiss vs. other wheat	1	1.7	0.002	1	0.7	0.006	1	1.8	0.171
3 conventional Swiss varieties	2	0.5	0.183	2	0.9	0.009	2	0.6	0.123
Bobwhite vs. Frisal	1	0.4	0.137	1	0.0	0.679	1	0.4	0.072
Bobwhite vs. Sb lines	1	0.0	0.959	1	0.1	0.182	1	0.0	0.840
Pm3b lines vs. Sb lines	1	2.7	<.001	1	2.4	<.001	1	3.1	<.001
4 Sb lines	3	1.3	0.044	3	0.1	0.724	3	1.2	0.035
4 *Pm3b* lines	3	1.3	0.047	3	1.7	0.001	3	1.2	0.038
A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.0	0.814	1	0.0	0.909	1	0.0	0.960
A9 *Chi* vs. A13 *Chi/Glu*	1	0.3	0.149	1	0.0	0.841	1	0.2	0.241
Plot	42	6.4	<.001	42	3.4	0.376	42	5.5	<.001
Fertilizer	1	17.2	<.001	1	9.7	<.001	1	17.0	<.001
Comp.env.×Fertilizer	14	0.5	0.596	14	0.8	0.709	14	0.4	0.696
Subplot	45	1.8	0.007	45	3.3	<.001	45	1.8	0.017
Phytometer contrasts:									
Swiss vs. other wheat	1	0.9	<.001	1	0.1	0.073	1	0.2	0.015
3 conventional Swiss varieties	2	0.1	0.204	2	0.0	0.658	2	0.1	0.068
Bobwhite vs. Frisal	1	0.3	<.001	1	1.1	<.001	1	0.6	<.001
Bobwhite vs. Sb lines	1	0.6	<.001	1	0.0	0.459	1	1.2	<.001
Pm3b lines vs. Sb lines	1	1.8	<.001	1	0.0	0.609	1	2.8	<.001
4 Sb lines	3	0.1	0.129	3	0.1	0.372	3	0.1	0.158
4 *Pm3b* lines	3	1.8	<.001	3	0.7	<.001	3	1.2	<.001
A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.0	0.441	1	0.0	0.647	1	0.1	0.053
A9 *Chi* vs. A13 *Chi/Glu*	1	0.3	<.001	1	0.0	0.759	1	0.4	<.001
Pairwise comparisons:									
Pm3b#1 vs. Sb#1	1	0.1	0.047	1	0.0	0.759	1	0.2	0.005
Pm3b#2 vs. Sb#2	1	2.1	<.001	1	0.1	0.095	1	1.8	<.001
Pm3b#3 vs. Sb#3	1	0.1	0.067	1	0.0	0.426	1	0.3	0.001
Pm3b#4 vs. Sb#4	1	0.4	<.001	1	0.1	0.104	1	1.1	<.001
A9 *Chi* vs. Frisal	1	0.0	0.293	1	0.0	0.582	1	0.0	0.698
A13 *Chi/Glu* vs. Frisal	1	0.2	0.017	1	0.0	0.795	1	0.4	<.001
Comp.env.×Phytometer lines	196	5.4	0.071	196	6.1	0.583	196	5.7	0.058
Plot×Phytometer lines	593	13.9	0.888	598	19.2	0.171	593	14.3	0.818
Fertilizer×Swiss vs. other wheat	1	0.0	0.946	1	0.0	0.287	1	0.0	0.534

Table S2 continues

Source of variation	Yield (log)			Spike number (log)			Seed number (log)		
	df	%SS	F pr.	df	%SS	F pr.	df	%SS	F pr.
Fertilizer×3 conventional Swiss varieties	2	0.0	0.964	2	0.1	0.438	2	0.0	0.798
Fertilizer×Bobwhite vs. Frisal	1	0.1	0.107	1	0.1	0.062	1	0.0	0.17
Fertilizer×Bobwhite vs. Sb lines	1	0.0	0.781	1	0.0	0.236	1	0.0	0.787
Fertilizer×*Pm3b* lines vs. Sb lines	1	0.0	0.415	1	0.0	0.503	1	0.0	0.642
Fertilizer×4 Sb lines	3	0.0	0.923	3	0.2	0.096	3	0.0	0.873
Fertilizer×4 *Pm3b* lines	3	0.1	0.486	3	0.1	0.613	3	0.1	0.369
Fertilizer×A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.0	0.253	1	0.1	0.128	1	0.1	0.152
Fertilizer×A9 *Chi* vs. A13 *Chi/Glu*	1	0.0	0.642	1	0.1	0.078	1	0.0	0.54
Residual	1406	36.0		1522	45.9		1406	36.2	
Total	2342	100		2463	100		2342	100.0	

Table S3. ANOVA table showing the effects of fertilizer, competitive environment, differences between GM and non-GM lines and their interactions on phenological stage, plant height and vegetative mass

Simple model

Source of variation	Vegetative mass (log)			Plant height (log)			Phenological stage (log)		
	df	%SS	F pr.	df	%SS	F pr.	df	%SS	F pr.
Block	3	2.2	0.005	3	31.8	<.001	3	28.1	<.001
Competitive environment	14	10.1	<.001	14	0.6	0.537	14	1.0	0.136
Plot	42	6.5	<.001	42	2.1	0.030	42	2.0	0.203
Fertilizer	1	27.7	<.001	1	7.0	<.001	1	5.1	<.001
Comp.env.×Fertilizer	14	0.4	0.904	14	0.9	0.017	14	0.4	0.674
Subplot	45	2.2	<.001	45	1.3	<.001	45	1.6	<.001
Phytometer lines	14	5.8	<.001	14	24.2	<.001	14	20.4	<.001
Comp.env.×Phytometer lines	196	3.6	0.896	196	2.0	0.846	196	2.4	0.999
Plot×Phytometer lines	597	12.7	0.082	630	7.4	<.001	630	11.8	<.001
Phytometer lines×Fertilizer	14	0.4	0.160	14	0.6	<.001	14	0.4	0.001
Residual	1473	28.5		2625	22.2		2594	26.9	
Total	2413	100		3598	100		3567	100	

Extended model

Source of variation	Vegetative mass (log)			Plant height (log)			Phenological stage (log)		
	df	%SS	F pr.	df	%SS	F pr.	df	%SS	F pr.
Block	3	2.2	0.005	3	31.8	<.001	3	28.1	<.001
Competitive environment contrasts:									
Swiss vs. other wheat	1	2.4	<.001	1	0.1	0.210	1	0.0	0.691
3 conventional Swiss varieties	2	1.0	0.050	2	0.1	0.489	2	0.2	0.089
Bobwhite vs. Frisal	1	0.3	0.175	1	0.1	0.200	1	0.4	0.004
Bobwhite vs. Sb lines	1	0.1	0.374	1	0.0	0.604	1	0.0	0.35
Pm3b lines vs. Sb lines	1	2.6	<.001	1	0.0	0.969	1	0.0	0.692
4 Sb lines	3	1.7	0.018	3	0.1	0.652	3	0.0	0.998
4 *Pm3b* lines	3	1.7	0.017	3	0.1	0.402	3	0.0	0.878
A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.0	0.703	1	0.1	0.115	1	0.3	0.025
A9 *Chi* vs. A13 *Chi/Glu*	1	0.2	0.297	1	0.0	0.399	1	0.0	0.921
Plot	42	6.4	<.001	42	2.1	0.030	42	2.0	0.203
Fertilizer	1	27.7	<.001	1	7.0	<.001	1	5.1	<.001
Comp.env.×Fertilizer	14	0.4	0.904	14	0.9	0.017	14	0.4	0.674
Subplot	45	2.2	<.001	45	1.3	<.001	45	1.6	<.001
Phytometer contrasts:									
Swiss vs. other wheat	1	0.0	0.395	1	6.0	<.001	1	3.3	<.001
3 conventional Swiss varieties	2	0.1	0.169	2	0.4	<.001	2	0.4	<.001
Bobwhite vs. Frisal	1	2.8	<.001	1	17.1	<.001	1	16.2	<.001
Bobwhite vs. Sb lines	1	0.1	0.013	1	0.0	0.224	1	0.0	0.111
Pm3b lines vs. Sb lines	1	0.2	<.001	1	0.2	<.001	1	0.2	<.001
4 Sb lines	3	0.3	<.001	3	0.0	0.515	3	0.0	0.591
4 *Pm3b* lines	3	2.2	<.001	3	0.4	<.001	3	0.1	0.023
A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.0	0.381	1	0.0	0.241	1	0.0	0.508
A9 *Chi* vs. A13 *Chi/Glu*	1	0.0	0.245	1	0.0	0.088	1	0.0	0.195
Pairwise comparisons:									
Pm3b#1 vs. Sb#1	1	0.0	0.015	1	0.0	0.599	1	0.0	0.187
Pm3b#2 vs. Sb#2	1	0.0	<.001	1	0.4	<.001	1	0.2	<.001
Pm3b#3 vs. Sb#3	1	0.0	0.768	1	0.0	0.060	1	0.0	0.139
Pm3b#4 vs. Sb#4	1	0.0	0.113	1	0.0	0.273	1	0.1	0.008
A9 *Chi* vs. Frisal	1	0.0	0.872	1	0.0	0.871	1	0.0	0.223
A13 *Chi/Glu* vs. Frisal	1	0.0	0.185	1	0.0	0.062	1	0.0	0.945

Table S3 continues

Source of variation	Vegetative mass (log)			Plant height (log)			Phenological stage (log)		
	df	%SS	F pr.	df	%SS	F pr.	df	%SS	F pr.
Comp.env.×Phytometer lines	196	3.6	0.896	196	2.0	0.846	196	2.4	0.999
Plot×Phytometer lines	597	12.7	0.082	630	7.4	<.001	630	11.8	<.001
Fertilizer×Swiss vs. other wheat	1	0.1	0.114	1	0.1	<.001	1	0.0	0.892
Fertilizer×3 conventional Swiss varieties	2	0.2	0.019	2	0.0	0.603	2	0.1	0.009
Fertilizer×Bobwhite vs. Frisal	1	0.1	0.095	1	0.3	<.001	1	0.1	0.012
Fertilizer×Bobwhite vs. Sb lines	1	0.0	0.694	1	0.0	0.842	1	0.0	0.196
Fertilizer×*Pm3b* lines vs. Sb lines	1	0.0	0.834	1	0.0	0.830	1	0.0	0.468
Fertilizer×4 Sb lines	3	0.0	0.820	3	0.1	0.024	3	0.0	0.443
Fertilizer×4 *Pm3b* lines	3	0.1	0.359	3	0.1	0.061	3	0.1	0.132
Fertilizer×A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.0	0.426	1	0.0	0.022	1	0.0	0.214
Fertilizer×A9 *Chi* vs. A13 *Chi/Glu*	1	0.0	0.319	1	0.0	0.503	1	0.1	0.004
Residual	1473	28.5		2625	22.2		2594	26.9	
Total	2413	100		3598	100		3567	100	

Table S4. ANOVA table showing the effects of fertilizer, competitive environment, differences between GM and non-GM lines and their interactions on three relative yield characteristics

Simple model

Source of variation	Yield (log)			Spike number (log)			Seed number (log)		
	df	%SS	F pr.	df	%SS	F pr.	df	%SS	F pr.
Overall mean	1	0.4	0.009	1	0.0	0.503	1	0.2	0.032
Block	3	1.4	0.021	3	1.9	0.001	3	1.8	0.002
Competitive environment	14	7.5	<.001	14	5.9	<.001	14	8.9	<.001
Plot	47	5.9	0.075	47	4.3	0.287	43	4.6	0.030
Fertilizer	1	0.5	0.014	1	0.1	0.290	1	2.3	<.001
Comp.env.×Fertilizer	14	0.6	0.917	14	0.3	0.991	14	0.3	0.977
Subplot	43	3.5	0.059	44	3.4	0.007	46	2.8	0.072
Phytometer lines	14	12.7	<.001	14	13.5	<.001	14	9.9	<.001
Comp.env.×Phytometer lines	180	6.7	0.999	180	6.8	0.999	182	6.8	0.997
Plot×Phytometer lines	502	35.6	0.029	520	40.6	<.001	552	29.2	0.031
Phytometer lines×Fertilizer	13	3.9	<.001	14	3.4	<.001	14	8.7	<.001
Residual	363	21.3		421	19.8		550	24.8	
Total	1195	100		1273	100		1433	100.0	

Extended model

Source of variation	Yield (log)			Spike number (log)			Seed number (log)		
	df	%SS	F pr.	df	%SS	F pr.	df	%SS	F pr.
Overall mean	1	0.4	0.009	1	0.0	0.503	1	1.8	0.002
Block	3	1.4	0.021	3	1.9	0.001	3	8.9	<.001
Competitive environment	14	7.5	<.001	14	5.9	<.001	14	4.6	0.030
Plot	47	5.9	0.075	47	4.3	0.287	43	2.3	<.001
Fertilizer	1	0.5	0.014	1	0.1	0.290	1	0.3	0.977
Comp.env.×Fertilizer	14	0.6	0.917	14	0.3	0.991	14	2.8	0.988
Subplot	43	3.5	0.059	44	3.4	0.007	46	1.8	0.002
Phytometer contrasts:									
Swiss vs. other wheat	1	1.1	<.001	1	0.2	0.022	1	0.7	<.001
3 conventional Swiss varieties	2	0.2	0.219	2	1.3	<.001	2	0.2	0.064
Bobwhite vs. Frisal	1	0.0	0.447	1	0.1	0.084	1	0.2	0.040
Bobwhite vs. Sb lines	1	0.8	<.001	1	1.2	<.001	1	0.1	0.116
Pm3b lines vs. Sb lines	1	5.7	<.001	1	4.4	<.001	1	4.4	<.001
4 Sb lines	3	1.3	<.001	3	1.5	<.001	3	1.1	<.001
4 *Pm3b* lines	3	3.5	<.001	3	4.7	<.001	3	2.9	<.001
A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.0	0.543	1	0.0	0.396	1	0.0	0.721
A9 *Chi* vs. A13 *Chi/Glu*	1	0.1	0.240	1	0.0	0.533	1	0.3	0.013
Pairwise comparisons:									
Pm3b#1 vs. Sb#1	1	1.5	<.001	1	0.3	0.010	1	0.6	<.001
Pm3b#2 vs. Sb#2	1	4.3	<.001	1	4.3	<.001	1	5.2	<.001
Pm3b#3 vs. Sb#3	1	0.2	0.066	1	0.1	0.156	1	0.2	0.065
Pm3b#4 vs. Sb#4	1	0.6	0.002	1	3.7	<.001	1	0.5	0.001
A9 *Chi* vs. Frisal	1	0.0	0.561	1	0.1	0.125	1	0.1	0.120
A13 *Chi/Glu* vs. Frisal	1	0.0	0.541	1	0.0	0.373	1	0.0	0.357
Comp.env.×Phytometer lines	180	6.7	0.999	180	6.8	0.999	182	6.8	0.997
Plot×Phytometer lines	502	35.6	0.029	520	40.6	<.001	552	29.2	0.031
Fertilizer×Swiss vs. other wheat	1	0.1	0.188	1	0.1	0.100	1	0.3	0.016
Fertilizer×3 conventional Swiss varieties	2	0.6	0.009	2	0.2	0.091	2	1.3	<.001
Fertilizer×Bobwhite vs. Frisal	1	0.1	0.257	1	0.0	0.340	1	0.0	0.568
Fertilizer×Bobwhite vs. Sb lines	1	0.6	0.001	1	0.0	0.647	1	0.8	<.001
Fertilizer×*Pm3b* lines vs. Sb lines	1	1.3	<.001	1	1.0	<.001	1	2.1	<.001
Fertilizer×4 Sb lines	2	0.6	0.005	3	0.7	0.002	3	3.1	<.001

Table S4 continues

Source of variation	Yield (log)			Spike number (log)			Seed number (log)		
	df	%SS	F pr.	df	%SS	F pr.	df	%SS	F pr.
Fertilizer×4 *Pm3b* lines	3	0.5	0.033	3	0.5	0.010	3	0.4	0.045
Fertilizer×A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.0	0.731	1	0.0	0.828	1	0.2	0.029
Fertilizer×A9 *Chi* vs. A13 *Chi/Glu*	1	0.0	0.803	1	0.7	<.001	1	0.6	<.001
Residual	363	21.3		421	19.8		550	24.8	
Total	1195	100		1273	100		1433	100.0	

Table S5. ANOVA table showing the effects of fertilizer, competitive environment, differences between GM and non-GM lines and their interactions on relative phenological stage, plant height and vegetative mass

Simple model

Source of variation	Vegetative mass (log)			Plant height (log)			Phenological stage (log)		
	df	%SS	F pr.	df	%SS	F pr.	df	%SS	F pr.
Overall mean	1	0.6	<.001	1	0.2	0.027	1	0.5	0.001
Block	3	0.4	0.439	3	1.0	0.045	3	0.2	0.619
Competitive environment	14	9.5	<.001	14	2.1	0.217	14	2.2	0.259
Plot	47	7.2	0.013	47	5.2	0.723	47	5.9	0.005
Fertilizer	1	0.6	0.007	1	0.0	0.730	1	0.3	0.021
Comp.env.×Fertilizer	14	0.3	0.994	14	1.2	0.823	14	1.4	0.085
Subplot	44	3.4	0.015	44	5.8	<.001	44	2.5	0.144
Phytometer lines	14	13.5	<.001	14	7.7	<.001	14	10.2	<.001
Comp.env.×Phytometer lines	180	5.2	0.999	180	5.5	0.999	180	4.9	0.999
Plot×Phytometer lines	515	35.9	<.001	610	33.3	0.025	610	32.4	0.040
Phytometer lines×Fertilizer	14	3.3	<.001	14	3.5	<.001	14	5.6	<.001
Residual	403	20.1		735	34.5		731	33.9	
Total	1250	100		1677	100		1673	100	

Extended model

Source of variation	Vegetative mass (log)			Plant height (log)			Phenological stage (log)		
	df	%SS	F pr.	df	%SS	F pr.	df	%SS	F pr.
Overall mean	1	0.6	<.001	1	0.2	0.027	1	0.5	0.001
Block	3	0.4	0.439	3	1.0	0.045	3	0.2	0.619
Competitive environment	14	9.5	<.001	14	2.1	0.217	14	2.2	0.259
Plot	47	7.2	0.013	47	5.2	0.723	47	5.9	0.005
Fertilizer	1	0.6	0.007	1	0.0	0.730	1	0.3	0.021
Comp.env.×Fertilizer	14	0.3	0.994	14	1.2	0.823	14	1.4	0.085
Subplot	44	3.4	0.015	44	5.8	<.001	44	2.5	0.144
Phytometer contrasts:									
Swiss vs. other wheat	1	0.7	<.001	1	0.0	0.336	1	1.6	<.001
3 conventional Swiss varieties	2	1.4	<.001	2	2.1	<.001	2	1.5	<.001
Bobwhite vs. Frisal	1	0.7	<.001	1	0.0	0.617	1	0.0	0.685
Bobwhite vs. Sb lines	1	1.2	<.001	1	0.2	0.066	1	1.3	<.001
Pm3b lines vs. Sb lines	1	3.3	<.001	1	0.4	0.005	1	0.3	0.012
4 Sb lines	3	0.5	0.026	3	2.9	<.001	3	3.0	<.001
4 *Pm3b* lines	3	5.4	<.001	3	1.4	<.001	3	0.2	0.295
A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.0	0.325	1	0.6	<.001	1	2.0	<.001
A9 *Chi* vs. A13 *Chi/Glu*	1	0.2	0.026	1	0.0	0.832	1	0.3	0.013
Pairwise comparisons:									
Pm3b#1 vs. Sb#1	1	0.4	0.006	1	0.1	0.210	1	0.9	<.001
Pm3b#2 vs. Sb#2	1	5.0	<.001	1	0.4	0.003	1	0.8	<.001
Pm3b#3 vs. Sb#3	1	0.0	0.523	1	0.6	<.001	1	0.3	0.009
Pm3b#4 vs. Sb#4	1	0.6	0.001	1	0.2	0.025	1	0.4	0.004
A9 *Chi* vs. Frisal	1	0.3	0.025	1	0.3	0.017	1	2.4	<.001
A13 *Chi/Glu* vs. Frisal	1	0.0	0.862	1	0.2	0.029	1	1.1	<.001
Comp.env.×Phytometer lines	180	5.2	0.999	180	5.5	0.999	180	4.9	0.999
Plot×Phytometer lines	515	35.9	<.001	610	33.3	0.025	610	32.4	0.040
Fertilizer×Swiss vs. other wheat	1	0.1	0.226	1	0.0	0.781	1	0.2	0.030
Fertilizer×3 conventional Swiss varieties	2	0.0	0.907	2	0.6	0.001	2	0.1	0.373
Fertilizer×Bobwhite vs. Frisal	1	0.0	0.716	1	0.3	0.013	1	0.1	0.235
Fertilizer×Bobwhite vs. Sb lines	1	0.8	<.001	1	0.0	0.544	1	0.6	<.001
Fertilizer×*Pm3b* lines vs. Sb lines	1	1.2	<.001	1	0.6	<.001	1	1.0	<.001
Fertilizer×4 Sb lines	3	1.0	<.001	3	0.1	0.631	3	0.4	0.025

Table S5 continues

Source of variation	Vegetative mass (log)			Plant height (log)			Phenological stage (log)		
	df	%SS	F pr.	df	%SS	F pr.	df	%SS	F pr.
Fertilizer×4 *Pm3b* lines	3	0.1	0.611	3	0.8	0.001	3	0.7	0.002
Fertilizer×A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.1	0.199	1	0.0	0.468	1	0.0	0.320
Fertilizer×A9 *Chi* vs. A13 *Chi/Glu*	1	0.0	0.324	1	1.1	<.001	1	2.4	<.001
Residual	403	20.1		735	34.5		731	33.9	
Total	1250	100		1677	100		1673	100	

CHAPTER 2

Post-Harvest Effects in a Field Trial with Transgenic Wheat: Persistence of Transgenic Seeds, Seedlings and Plants and Effects on Fallow Weed Communities

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Wheat plants persisting in the field in January 2009

Abstract

Introduction of transgenic crops to agriculture has raised concerns about their possible effects on agro-ecosystems. We compared nine conventional lines of spring wheat with six genetically modified (GM) lines that contained transgenes of resistance against powdery mildew (*Pm3b* gene) or against fungi in general (*chitinase* and *glucanase* genes). We assessed the persistence of these lines without competition and in experimental weed communities in the field, their germination in the laboratory, their survival in fallow plots in the field, and their effects on post-harvest vegetation in the field. Planted under competition in experimental weed communities, the GM plants showed weaker performance than their conventional counterparts. No such performance differences were observed without competition in the field. The seeds and seedlings of GM lines did not persist longer than those of the corresponding non-GM lines in fallow plots. The lowest seed and biomass output and lowest seed germination rate in the laboratory was observed in the GM line with the highest transgene expression. We argue that this might be due to the costs incurred by the introduced constitutive resistance to pathogens or a pleiotropic effect of the transgene. Although GM plants did not perform better or persist longer compared to their conventional counterparts, they were able to reproduce in dense weed communities and to successfully survive winter on fallow plots. The seeds of both GM and conventional wheat lines either germinated shortly or lost their viability already after 3 months of storage in soil in the laboratory. Poor seed longevity in the laboratory yet successful plant persistence in weed communities or on fallow plots indicate that not removing a population of growing plants presents a greater risk than allowing the build-up of a soil seed bank regarding the potential escape of GM wheat to the environment. However, such persisting GM plants had no apparent effects on the structure and diversity of fallow plant communities.

Introduction

Genetically modified (GM) crops have been widely adopted, with over 107 km^2 cultivated in 29 countries worldwide in 2010 (James 2010). Development of GM crops, however, has raised concerns about possible persistence of transgenes in agro-ecosystems (Linder and Schmitt 1994, Purrington and Bergelson 1995, Snow 2002, Warwick *et al.* 2009). New introduced traits, in particular those that confer resistance to pathogens or abiotic stress, can potentially increase weediness or invasiveness of the GM plants or their offspring within agricultural, uncultivated, or natural areas (Schmitt and Linder 1994, Purrington and Bergelson 1995, Hails 2000, Andow and Zwahlen 2006, Warwick *et al.* 2009). Seed persistence in soil and GM plant volunteering in subsequent conventional crops can result in unintended contamination of non-GM seed lots (Friesen *et al.* 2003, Demeke *et al.* 2006, Mallory-Smith and Zapiola 2008). Another concern is that GM crops might have indirect environmental effects on biodiversity in fallow fields and might alter the species composition of weed communities (Harker *et al.* 2005b, Culpepper 2006, Warwick *et al.* 2009).

Wheat is the most important food crop in temperate climate with 682.5 million tons world production forecast for 2011 (FAO 2011a, b). Although transgenic wheat is not yet widely commercialized, a range of traits has already been introduced into wheat plants, including drought tolerance (Sivamani *et al.* 2000a, Bahieldin *et al.* 2005), insect (Altpeter *et al.* 1999, Stoger *et al.* 1999) and disease resistance (Altpeter *et al.* 1999, Sivamani *et al.* 2000b, Bieri *et al.* 2003, Brunner *et al.* 2011) and grain quality (Vasil *et al.* 2001). Many countries are currently fast-tracking the development of GM wheat varieties, the first of which are expected to be ready for commercialization in 2017 (James 2010).

We used nine non-GM and six GM lines or varieties (later simply called lines) of spring wheat *Triticum aestivum* L. to study the persistence of plants of these lines in the environment at different stages of their life cycle. The GM lines were genetically modified to be resistant against powdery mildew *Blumeria graminis* f.sp. tritici (DC.) Speer or had a general quantitative resistance against fungi. From 2008–2009, we carried out a series of experiments to assess the performance of the non-GM and GM lines without competition and in two different experimental weed communities in the field, their seed longevity in the soil in the laboratory, seedling volunteering, persistence and over-winter survival in fallow plots in the field and the potential effects of these plants in fallow plots on post-harvest weed communities.

The work presented here is part of a joint project of several research groups called “wheat cluster” (www.wheat-cluster.ch) which used wheat as a model species to compare non-GM with GM lines within the framework of the Swiss National Research Program 59 “Benefits and risks of the deliberate release of genetically modified plants” (www.NRP59.ch). Other projects within the “wheat cluster” studied transgene × environment interactions in the glasshouse and in the field (Zeller *et al.* 2010, Kalinina *et al.* 2011), disease resistance and transgene expression (Brunner *et al.* 2011) and the impact of the transgenic wheat lines on associated organisms at other trophic levels (von Burg *et al.* 2010, Alvarez-Alfageme *et al.* 2011).

We asked the following questions: (1) how do non-GM and GM plants perform without competition and in experimental weed communities common for wheat fields and wheat fallows? (2) Can the seeds of wheat persist in soil throughout winter and is seed longevity different between GM and conventional lines? (3) Do GM seedlings appear more often and do they persist longer than those of conventional wheat on post-harvest fallow plots? (4) Are there any post-harvest effects of wheat plants on fallow weed communities?

We found that the GM wheat lines had weaker performance compared to the control lines when grown in weed communities — an effect not observed in the absence of competitors. There was no indication that the GM lines had higher seed or seedling persistence than the control lines, and no effects on post-harvest weed communities were found.

Materials and Methods

Plant material

We used six transgenic lines derived from two maternal varieties of spring wheat, the Mexican variety Bobwhite and the old Swiss variety Frisal. These two varieties were chosen because they show high transformation efficiency and are susceptible to powdery mildew pathogen (Pellegrineschi *et al.* 2002, Bieri *et al.* 2003). Four transgenic lines (*Pm3b*#1–4) were produced by biolistic transformation of Bobwhite in different transformation events. *Pm3b*#1–3 lines carried a single copy of the transgene *Pm3b,* and *Pm3b*#4 line carried one full-length and one inactive truncated copy (Brunner *et al.* 2011). Their respective non-transgenic sister lines Sb#1–4 (null-segregants) were used as a control to ensure that any somaclonal variations acquired during tissue culturing were shared between transgenic and control lines. The *Pm3b*

gene confers race-specific resistance to powdery mildew and was cloned from hexaploid wheat (Yahiaoui *et al.* 2004). The seeds used in this study were obtained from homozygous GM and control lines that had passed through five generations of sexual reproduction. The ubiquitin promoter from maize ensured high transgene expression.

The GM lines derived from the variety Frisal expressed a barley seed *chitinase* (line A9 *Chi*) or *chitinase* and *β-1,3-glucanase* genes (line A13 *Chi/Glu*) (Leah *et al.* 1991, Bieri *et al.* 2003). The expression of these pathogenesis-related genes should result in increased quantitative resistance to fungi, including mildew (Zhu *et al.* 1994). The seeds used for the experiment were obtained from the sixth generation of transgenic lines A13 *Chi/Glu* and A9 *Chi*. Wheat variety Frisal was used as a control.

In addition to the 11 lines or varieties already mentioned, we used variety Bobwhite (plants that had not passed through tissue culture) and three commercialized conventional wheat varieties Casana, Fiorina and Toronit (in the following "lines and varieties" are referred to as "lines") as reference to verify whether the characteristics of the GM plants fall within the range of variation between conventional varieties of wheat, the criterion of the test of equivalence required by the European Food Safety Authority for risk assessment of GM plants (EFSA 2011).

Performance of the wheat plants in weed communities

This field experiment took place in 2008 at ART Reckenholz research station in Zurich, Switzerland. In March 2008, two types of weed mixtures typical (1) for agricultural wheat fields and (2) for wheat-fallow fields (Table S1 in Supplemental Material) were sown in eight 7×1.08 m plots arranged in a randomized complete block design with four replicate blocks. In addition, one plot per block was left bare (no-competition environment). The plots were split into subplots. The two 1×1.08 m edge subplots of each plot were used for a split-plot treatment, i.e. fertilizer application vs. control. Fertilizer was applied twice during the growing season (two times 3 g N m^{-2} as "Ammonsalpeter 27.5", Lonza, Visp, Switzerland) to one of the two subplots in each plot.

In February 2008, 720 individual seeds of the 15 lines of *T. aestivum* (see "Plant material" section) were germinated in a climate-controlled glasshouse (day/night temperature: 21/16 C°; additional light: 14 h/10 h day/night period). In March 2008, the seedlings at phenological stage 11 (i.e. the first leaf unfolded; Zadoks *et al.* 1974) were

transplanted from the glasshouse to the field subplots with two different weed communities or empty subplots (no-competition control). These seedlings grown under standard conditions in the glasshouse were used to assess the performance of the GM and non-GM wheat lines in weed communities under two soil nutrient levels. Thirty seedlings representing 15 lines of *T. aestivum* were introduced into each 1×1.08 m subplot. The seedlings were planted into six rows with a distance of 18 cm between the rows and 20 cm between the neighboring wheat plants in a row (Figures S1, S2 in Supplemental Material). Each wheat line was represented twice in each subplot (see also Kalinina *et al.* 2011). The naturally emerging weeds were regularly removed from the no-competition control plots but were left to grow in the weed mixture plots. These, however, had much lower abundance than the species sown intentionally. In June 2008, the weed species growing in the weed-mixture plots were identified. The lists of the weed species sown and those that naturally emerged are shown in Table S1 in Supplemental Material.

We recorded the phenological stage of all plants according to the "Zadoks" scale (Zadoks *et al.* 1974) 80 days after planting. The incidence of powdery mildew infection was also assessed 80 days after planting, when infection reached its maximum. For each wheat line in each treatment we calculated a percentage of the plants infected with the pathogen and used this as a dependent variable in subsequent analyses. After ripening, all wheat plants were cut at ground level and separated into vegetative and reproductive parts (spikes). The plant material was dried at 80 C° (vegetative parts) or 25 C° (reproductive parts) and weighed. We threshed the spikes, determined the seed number per plant and obtained the total mass of all seeds per plant. The biomass allocation to seeds was calculated as a percentage of aboveground plant biomass. Henceforth, the seed number per plant is called "seed number" and biomass allocation to seeds is called "biomass allocation".

Data were analyzed with classical mixed-model analysis of variance (ANOVA) using the statistical software GenStat (GenStat v13.1.44, VSN International Ldt.). The treatment model consisted of the factorially-crossed wheat lines and environments (i.e. two weed mixtures and no-competition control) and fertilizer application. The error model consisted of the wheat lines nested within subplots, subplots nested within plots and plots nested within blocks. The terms of the treatment model were tested against the appropriate terms of the error model: environment varied among plots, fertilizer application among subplots and wheat line within subplots. For the analysis, the seed

number data were log-transformed. The data were analyzed for all the environments together to test the main effects and also for weed environments and for no-competition environments separately. The structure of the comparisons for the alternative models used is shown in Figure S3 in Supplemental Material. The binary mildew incidence data were analyzed using multiple logistic regression with mixed-model analysis of deviance (McCullagh and Nelder 1989).

Seed persistence in the soil

The seeds of the 15 wheat lines for this experiment were obtained from the NRP59 field trial 2008 (Zeller *et al.* 2010). Half of the seeds were from the plants which did not receive fertilizer in the field and the other half from the plants fertilized twice during the growing season 2008 (3 g N m^{-2} as "Ammonsalpeter 27.5", Lonza, Visp, Switzerland). To assess seed persistence in soil, the seeds of the wheat lines were stored in a ventilated chamber in the laboratory under conditions resembling soil conditions in the field in winter (T = 6^0C, complete darkness) for three or for 6 months. Before setting up the seed persistence experiment, initial germination rates were tested in Petri dishes in five replicates in the glasshouse. Twenty seeds were placed into each Petri dish (Ø 10 cm) on filter paper resulting in 100 seeds per wheat line in total. The Petri dishes were randomized and watered regularly. Germination rates were recorded every three days as a percentage of seeds germinated.

For the seed persistence experiment, 20 seeds were placed into each Petri dish filled with dry (17.6% humidity) and wet (77.5% humidity) Ökohum lawn soil (Ökohum AG, Herrenhof, Switzerland). Half of the seeds were kept in aerobic and another half in anaerobic conditions. To maintain anaerobic conditions, the Petri dishes were placed into polyethylene airproof bags sealed with vacuum sealer. Thirty Petri dishes (15 wheat lines × two mother-plant nutrient levels) were placed into each bag together with two sulfur-free oxygen absorbers (ATCO FTM 2000S, Long life for art, Germany). The air in the bags was replaced by nitrogen dioxide with a water jet vacuum needle, and then nitrogen dioxide was also removed. For the aerobic treatment, the bags with Petri dishes were left open. Half of the bags were stored for 3 months and another half for 6 months, the approximate time after harvest needed for seeds to germinate in autumn or in spring, in the laboratory. Altogether, there were five replicate Petri dishes and 100 seeds per mother-plant nutrient level × wheat line × time of storage × storage condition combination. After 3 and 6 months, respectively, the seeds were

removed from Petri dishes, washed and the number of seeds germinated during storage was recorded. Seeds that did not germinate during storage were placed into new Petri dishes for germination tests, performed in the same way as the initial germination tests.

Monitoring of seedling persistence in the field

From March until August 2008, the 15 wheat lines (see Plant material section) were grown in 120 field plots of 1×1.08 m size at ART Reckenholz research station in Zurich, Switzerland. In each plot 400 wheat seeds were sown in six rows with a distance of 18 cm between rows (Zeller *et al.* 2010). The plots were arranged in four blocks. Half of the plots were fertilized twice during the growing season (3 g N/m^2 as "Ammonsalpeter 27.5", Lonza, Visp, Switzerland), the other half was left untreated. In August 2008, the plants were harvested by hand (cut at soil level) and shed seeds and spikes were collected from the soil surface. Starting from the end of September 2008 and until the end of March 2009, we monitored the emergence of wheat seedlings from overlooked and thus uncollected seeds and followed their growth and survival. We distinguished three categories of "the emergence events": the seedlings that emerged from single seeds ("individual seedlings"), the whole spikes germinated ("patches of seedlings") and new tillers coming from the plants cut at a ground level. Since it was not possible to distinguish individual plants in a dense patch, each germinated spike was counted as a single emergence event. In case of new tillers, each individual plant which formed a new tiller was counted as an individual emergence event. In September 2008, all the emergence events were counted and individual seedlings (i.e. emerged from single seeds) were marked with plastic labels indicating the date of count. In plots where more than 15 individual seedlings emerged, only 15 of them were randomly selected and marked. During the following five census counts in October, November, January, February and March we recorded the number of the marked individual seedlings that persisted in the field.

Seedling mortality data were analyzed with generalized linear mixed-effects models (GLMM) with binomial errors and logit link using GenStat software (GenStat v13.1.44, VSN International Ldt.). Seedling mortality rates presented in figures were calculated as a percentage of dead seedlings at the end of a time interval out of those that were still alive at the beginning of the interval. During all the census counts we also counted the individual seedlings, patches of seedlings and tillers which emerged after the initial count in September 2008.

Vegetation analysis of the post-harvest weed communities

In November 2008 and in March 2009, we assessed and analyzed the weed communities established in the subplots where 15 different GM and conventional wheat lines were grown from spring–summer 2008 (the same 1.08 m^2 subplots used for the monitoring of seedling persistence). Species richness (the number of species present), species abundance according to a multilevel scale (Braun-Blanquet 1932) and total canopy cover in percentage were recorded for every subplot. For the analysis, the original Braun-Blanquet scale was transformed to cover percentage, with total species cover values ranging from 0.6 to 160% (van der Maarel 2007). Shannon-Wiener diversity indices (Kent and Coker 1992) were calculated for every subplot. The post-harvest effects of the wheat lines and of fertilization on species richness, species abundance, total canopy cover and the abundance of the four dominant weed species were investigated with mixed-model ANOVAs (see previous section).

The binary data of the occurrence of the four dominant species were analyzed with mixed-model analysis of deviance. The composition of the entire weed community was analyzed with principal coordinate analysis (PCO) of Euclidean and Jaccard similarity matrices with GenStat software (GenStat v13.1.44, VSN International Ldt.).

Results

Plant performance in weed communities

Mildew incidence was 1.6-fold lower when wheat plants were grown with competitors (weeds) than when planted alone (no competition vs. weed competition contrast: P=0.042; Figure 1, Table S2 in Supplemental Material), thus, indicating a protective effect of neighbors not susceptible to the pathogen. Transgenic *Pm3b* lines showed up to five-fold lower mildew incidence than corresponding control Sb lines (*Pm3b* vs. Sb lines contrast: P<0.001). All the four *Pm3b* lines had significantly lower mildew incidence than their corresponding sister lines. A line with high transgene expression, *Pm3b*#2, showed especially low mildew incidence while grown without competition and was not infected at all in weed communities. Three commercial Swiss wheat varieties Casana, Fiorina and Toronit had lower mildew incidence than the other varieties (Swiss vs. other wheat contrast: P<0.001). Frisal transgenic and conventional lines, overall, were less infected with mildew than the lines derived from the mildew-susceptible variety Bobwhite (Bobwhite vs. Frisal contrast: P<0.001).

Transgenic lines A9 *Chi* and A13 *Chi/Glu* had lower mildew incidence than their mother variety Frisal when grown without competition (A9 *Chi* and A13 *Chi/Glu* vs. Frisal contrast: P=0.004); line A9 *Chi* was not infected and line A13 *Chi/Glu* had 3.5-fold lower mildew incidence than the Frisal mother variety (Figure 1). None of the A9 *Chi*, A13 *Chi/Glu* or Frisal plants were infected when planted in weed communities. Fertilizer application increased overall mildew incidence 2.5-fold (main fertilizer effect: P=0.013).

The canopy cover in all the plots sown with the weed mixture was 100% thus creating a strong competitive environment for the wheat plants. Weed competition caused an overall 2.2-fold decrease in seed number and 1.8-fold decrease in tiller number of the wheat lines compared to the control subplots without competition (no-competition vs. weed competition contrast: P=0.001 for seed number, P=0.004 for tiller number).

Transgenic *Pm3b* lines had overall a 1.6-fold lower seed number (*Pm3b* vs. Sb lines contrast: P<0.001), 1.1-fold lower biomass allocation (P=0.013) and 1.1-fold lower plant height (P=0.006) than the Sb sister lines. These differences were more pronounced in weed environments (*Pm3b* vs. Sb lines contrast in weed environments: P=0.031 for plant height, P<0.001 for seed number, P=0.002 for biomass allocation) and were insignificant (*Pm3b* vs. Sb lines contrast in no-competition environment: P=0.084 for plant height, P=0.37 for biomass allocation) or less pronounced (P=0.004 for seed number) when plants were grown without competition. Only line *Pm3b*#4 had significantly lower seed number than its control when grown in no-competition plots (*Pm3b*#4 vs. Sb#4 contrast in no-competition environment: P<0.001). The other three *Pm3b* lines had reduced seed number compared to corresponding sister lines only when planted in weed communities: *Pm3b*#1 showed 2-fold reduced seed number (*Pm3b*#1 vs. Sb#1 contrast in weed environments: P=0.01), *Pm3b*#2 showed 2.8-fold (*Pm3b*#2 vs. Sb#2 contrast: P<0.001) and *Pm3b*#4 showed 2.6-fold reduced seed number (*Pm3b*#4 vs. Sb#4 contrast: P=0.003).

None of the *Pm3b* lines differed from sister lines in tiller number and in plant height when planted without competition. In weed communities, however, line *Pm3b*#2 had 1.4-fold reduced tiller number compared with line Sb#2 (*Pm3b*#2 vs. Sb#2 contrast: P=0.046); line *Pm3b*#1 had 1.2-fold and line *Pm3b*#2 had 1.1-fold reduced plant height compared with corresponding sister lines (*Pm3b*#1 vs. Sb#1 contrast: P=0.004; *Pm3b*#2 vs. Sb#2 contrast: P=0.05). There were no differences in

phenological stage between GM and control wheat lines, with the exception of line *Pm3b*#4, which had less advanced phenological stage than its corresponding sister line in control plots (*Pm3b*#4 vs. Sb#4 contrast in no-competition environment: P<0.001): 25.7 for *Pm3b*#4 compared to 28.6 on Zadoks scale for Sb#4 line.

The four *Pm3b* wheat lines differed significantly in their performance, namely in their seed number, plant height and phenological stage (P=0.004, P=0.003, P=0.039 for seed number, plant height and phenological stage, respectively). Overall, the lines *Pm3b*#2 and *Pm3b*#4 had lower seed number than the other two GM lines. *Pm3b*#2 showed also lower plant height than the other *Pm3b* lines. The differences in seed number, biomass allocation and plant height between *Pm3b* and Sb lines exceeded those between the three commercialized Swiss wheat varieties (%SS in Tables S2-4 in Supplemental Material).

Transgenic lines A9 *Chi* and A13 *Chi/Glu* did not show significant differences in their performance from the mother variety Frisal.

Fertilizer application led to a 1.4-fold increase in seed number, a 1.2-fold increase in plant height and a 1.5-fold increase in tiller number (fertilizer effect: P=0.025, P=0.001 and P<0.001 for seed number, plant height and tiller number, respectively) and did not affect biomass allocation and phenological stage of the wheat plants.

Seed persistence in the soil

The initial seed germination ability (before storage) did not differ between GM and control wheat lines. The average initial germination rate was 97.4%. Only Swiss variety Fiorina had lower initial germination rate than the other lines and varieties (91%; 3 Swiss varieties contrast: P=0.007 for initial germination ability).

On average, 87.0% of the seeds stored under different conditions germinated during storage. The seeds that did not germinate during storage also did not germinate later in the germination tests and were considered to be not viable. More seeds (91.0%) germinated in the Petri dishes stored for a period of 6 months than in those stored for 3 months (83.0%; time of storage main effect: P<0.001; Table S5 in Supplemental Material). Although the seeds germinated in both aerobic and anaerobic conditions, a higher germination rate (97.2%) was observed in aerobic than in anaerobic conditions (76.7%; aerobic vs. anaerobic conditions contrast: P<0.001). Moreover, the roots were

growing for a longer period of time and were, according to visual observations, longer under aerobic than under anaerobic conditions.

More seeds germinated in dry soil than in wet soil (soil humidity main effect: $P<0.001$). In aerobic conditions water availability played a positive role in seed germination: more seeds germinated in wet soil than in dry soil. In anaerobic conditions, however, water caused a decrease in seed germination (interaction aerobic vs. anaerobic condition × soil humidity: $P<0.001$; Table S5).

Whether the maternal plants received additional nutrients or not (main fertilizer effect) did not affect the initial germination ability of the seeds or their germination during storage (Table S5).

Overall, we found no differences in seed persistence between GM and non-GM lines. The seeds of the line *Pm3b*#2, however, had a significantly lower germination rate (92.2%) than the corresponding control line Sb#2 (98.2%) under aerobic dry conditions of storage (*Pm3b*#2 vs. Sb#2 line contrast in aerobic conditions: $P<0.001$; interaction *Pm3b*#2 vs. Sb#2 × Soil humidity in aerobic conditions: $P=0.015$), but this effect was not observed under other storage conditions.

The three conventional Swiss wheat varieties differed in seed germination during storage (3 Swiss varieties contrast: $P<0.001$). Overall, Fiorina and Toronit had lower seed germination rate during storage in soil (84.8% and 87.5%, respectively) than the variety Casana (92.5%). There were also varietal differences in the response to oxygen content in soil (interaction 3 Swiss varieties × aerobic vs. anaerobic conditions: $P=0.013$). Under aerobic conditions, the three varieties had similar germination rates (96.4%, 95.6% and 97.4% for Toronit, Fiorina and Casana, respectively), whereas under anaerobic conditions Fiorina showed 8.9% lower germination ability compared to the other two Swiss wheat varieties (interaction 3 Swiss varieties × aerobic vs. anaerobic conditions: $P<0.001$).

Seedling persistence in the field

In total 1053 emergence events, i.e. individual seedlings, germinated patches of seedlings and new tillers, occurred in the field during the period of monitoring. Of these emergence events 88.6% were individual seedlings coming from single seeds, 6.4% were whole spikes germinated (the number of patches of seedlings was counted), and 5% were new tillers coming from the base of harvested plants cut at ground level. The vast majority of all the emergence events (87.3%) occurred already in September 2008,

one month after harvest, 10.7% occurred in October 2008 and 1.3% in November 2008. Three months after harvest we observed no further plant emergence. During the period of monitoring, 32 wheat plants which persisted in the field formed spikes; 87.5% of these developed spikes within the first two months after harvest and the rest formed spikes in the following spring. Twelve out of the 32 plants which developed spikes were transgenic.

The number of tillers and germinated patches of seedlings did not differ between GM and control plots. However, 25.9% more individual seedlings emerged in the plots where control Sb lines were grown than in those with *Pm3b* transgenic lines (*Pm3b* vs. Sb lines contrast: P=0.018). As the plots sown with GM wheat were harvested first due to safety requirements, this could have affected the number of shed seeds from GM and control plots. Three commercialized Swiss wheat varieties Casana, Fiorina and Toronit differed in their post-harvest tiller development (3 Swiss wheat varieties contrast: P<0.001): variety Fiorina showed lower new tiller emergence than the other two varieties. Fertilization applied during the field season 2008 did not influence the number of the emergence events. Among all the emergence events, the highest overwinter survival was observed for the patches of seedlings from germinated spikes: 82.1% of the patches of seedlings persisted until March 2009. The overwinter survival of the tillers and all the emerged individual seedlings was 52.8% and 56.3%, respectively.

The individual seedlings that emerged in the field in September 2008 were labeled (up to 15 plants per plot; see Materials and methods section) and their persistence was surveyed until March 2009. Seedling mortality logically increased with the length of the observation interval (days between counts effect: P<0.001; Table S6 in Supplemental Material) and showed a rapid increase 161 days after harvest, i.e. at the fifth monitoring count on January 24, 2009 (time effect: P<0.001; Figures 2A and 2B). This increase in mortality coincided with lower air and soil temperatures and a decrease in precipitation during the winter months (Figure 2C).

The seedlings of the three modern Swiss varieties showed significantly lower mortality rates than the average of the other lines (Swiss vs. other wheat contrast: P=0.002). Among those, the Mexican variety Bobwhite and the lines derived from it had much higher mortality in the field than the Swiss variety Frisal and its two GM lines A9 *Chi* and A13 *Chi/Glu* (Bobwhite vs. Frisal contrast: P<0.001). Transgenic lines did not differ from control lines in seedling mortality rates (*Pm3b* vs. Sb lines

contrast: P=0.279, A9 *Chi* and A13 *Chi/Glu* vs. Frisal contrast: P=0.537; Table S6 in Supplemental Material).

Of the total of 810 individual seedlings which were labeled in September 2008 and surveyed, 53.3% (432) survived winter and persisted in the field until the time of the last monitoring in March 2009. Among the surveyed individual seedlings, there were 193 *Pm3b* GM plants and 33.7% of these persisted until March 2009.

The number of emerging control Sb seedlings was 256 and 42.2% of them persisted until March 2009. The number of emerging transgenic Frisal seedlings was 98 and 76.5% of them persisted until March. Among the 63 individual seedlings of control Frisal line which were labeled, 71.4% persisted until the end of monitoring.

Post-harvest weed vegetation

We found 47 species growing in the plots in autumn 2008 and 30 species in spring 2009 (Table S7 in Supplemental Material). The dominant species were *Seneceo vulgaris* L., *Poa annua* L. and *Veronica persica* Poir. with 2.5, 1.9 and 1.7% average plot canopy cover, respectively. In autumn, *P. annua* was more abundant in previously fertilized plots (P=0.035). There were no other effects of fertilization, wheat line or the time of count on the abundance of the dominant species.

The principal coordinate analysis (PCO) of Euclidean distances and Jaccard similarity matrices of the two vegetation counts showed no difference in vegetation composition between the plots where transgenic lines had been grown and those where conventional wheat lines had been grown prior to the monitoring (Figure 3). Fertilizer application during the vegetation season 2008 also had no effect on post-harvest weed community composition in the plots. Although the PCO of Euclidean distances showed a clustered pattern, this clustering was not due to the wheat line, fertilizer application or block effects.

Species richness, Shannon-Wiener diversity and total canopy cover did not differ between the plots previously planted with transgenic lines or conventional wheat lines (Tables S8-10 in Supplemental Material).

In November 2008, three months after harvest, there was a marginally significant effect of fertilization on species richness (main fertilizer effect: P=0.063) indicating a 6.8% lower species number in fertilized plots. This effect disappeared by the next vegetation analysis in April 2009.

Discussion

Plant performance in weed communities

The first question in the Introduction asked whether transgenic wheat plants have a better performance than conventional ones in the field when grown without competition or in experimental weed communities common for wheat fields or wheat fallows. Both GM and non-GM wheat plants had weaker performance under competition with weeds than when they were grown without competitors. The GM plants carrying the *Pm3b* transgene, however, showed fitness reductions compared to control plants when grown in the weed communities and did not differ or differed less from the controls when grown alone. These results correspond well with results of previous assessments of the performance of the same wheat lines under competition in different wheat crop stands (Kalinina *et al.* 2011): under stronger competition the fitness disadvantages of resistant GM plants became more evident. Our finding supports the view that costs of resistance can be more apparent under conditions stressful for the plant, i.e. environmental stress or competition from neighbors (Dewitt *et al.* 1998, van Dam and Baldwin 2001). As shown by Dewitt *et al.* (1998), the costs are more likely to be seen when plants deploy several phenotypic responses simultaneously, so that internal resource trade-offs limit performance. In our study, lower reproductive output of GM plants under competition with weeds could possibly be due to the simultaneous response of the wheat plants to competition and the constitutive expression of pathogen defense, which diverted resources from the processes involved in reproduction (Dewitt *et al.* 1998, Tollrian and Harvell 1999).

The seed number reduction and reduced biomass allocation to seeds were more pronounced in the GM line *Pm3b*#2 with the highest transgene expression, whereas the line *Pm3b*#3 known for segregation in resistance did not differ from the corresponding control line. This supports the idea that the magnitude of the cost of resistance could be related to the level of expression of the transgene (Zeller *et al.* 2010, Kalinina *et al.* 2011). Transgenic plants neither grew better nor produced more seeds when planted into weed communities compared to corresponding non-GM wheat lines. Therefore the risk for these plants to persist among the weeds is not higher than for conventional wheat varieties. Transgenic plants, however, along with non-GM plants, were able to grow and successfully reproduce even under conditions of a 100% weed canopy cover. Thus, if GM wheat plants would germinate from seeds in the field after harvest or escape from an agricultural field to natural habitats, they would have the potential to

reproduce and possibly persist in weed communities. A thorough control of the GM-wheat-fallow fields and adjacent areas, therefore, would be advisable. It is believed that GM wheat poses only low risks to spread and persist in natural habitats in Europe because it has no close wild relatives (except *Aegilops cylindrica* Host.), does not have a persistent soil seed bank and is a predominantly self-pollinating species (Hancock 2003). Our results, however, indicate that wheat generally can grow in weedy habitats and has a potential to persist among weeds, for at least one vegetation season, and produce seeds. To assess how long it could persist in such habitats, multi-year experiments would be necessary where the emergence and growth of volunteering wheat offspring could be continuously monitored.

Seed persistence in the soil

The second question we asked was whether seeds of GM wheat can persist in soil throughout winter and whether the seed longevity is different for GM and conventional wheat lines. We found no indication that seeds of the studied GM lines could persist longer in soil than the seeds of their corresponding control lines at given humidity, oxygen and temperature conditions. Studies on other GM crops, such as herbicide-tolerant oilseed rape, for instance, also showed that GM lines had no advantage compared to conventional varieties in their seed persistence in soil (Gruber *et al.* 2004, Lutman *et al.* 2005). Moreover, in our study one of the GM lines (*Pm3b*#2) known for high transgene expression (Zeller *et al.* 2010, Brunner *et al.* 2011, Kalinina *et al.* 2011) showed lower seed germination than its corresponding control line when the seeds were stored under aerobic dry conditions. Lower germination rates were also reported for transgenic rape in a seed burial study (Hails *et al.* 1997). One of the explanations might be physiological costs associated with the constitutive transgene expression or pleiotropic effects of the transgene which could potentially affect seed viability. For the wheat line *Pm3b*#2 we have previously observed and reported other unintended phenotypic changes, such as chlorophyll deficiency, weaker competitive performance, lower seed set and reduced agronomic yield (Zeller *et al.* 2010, Brunner *et al.* 2011, Kalinina *et al.* 2011).

In our experiment, most of the seeds either germinated quickly or lost their viability already after 3 months of storage in soil. This supports the results of some other studies which have shown that a persistent seed bank in soil is not common for wheat cultivars (Harker *et al.* 2005a, Nielson *et al.* 2009). Nielson *et al.* (2009), for

example, reported a 99% loss of seed viability for conventional cultivars of Canadian spring wheat within 6 months after seed burial in the field.

Soil oxygen conditions and humidity played an important role for seed germination in our experiment. More seeds germinated under aerobic conditions than under oxygen shortage and more seeds germinated in dry soil than in wet soil. This might be related to the oxygen shortage in wet soil, whereas 17.6% of soil humidity in the dry-soil treatment was sufficient for seeds to germinate. Water excess in soil was advantageous for the germination of wheat seeds in aerobic conditions but disadvantageous under oxygen shortage. Some studies suggest that wheat is especially sensitive to anaerobic conditions (Menegus *et al.* 1991) and wheat seeds are not able to germinate or the roots die fast after germination under anoxia (Morinaga 1926, Perata *et al.* 1992). Germination of the seeds in our anaerobic treatment could be due to residual oxygen left in soil in Petri dishes which apparently was enough for seeds to germinate but precluded further growth of seedlings, as, according to our visual observations, the roots were longer in aerobic than under anaerobic conditions.

Seedling persistence in the field

Our third question was whether GM seedlings appear more often and persist longer than those of conventional wheat in post-harvest weed communities. To address this question we assessed the emergence and persistence of individual wheat seedlings which emerged in the field after harvest. Volunteering wheat plants, however, were represented not only by seedlings coming from individual seeds lost at harvest. Therefore, we additionally assessed the persistence of dense patches of seedlings germinating from whole spikes (the number of patches persisting was counted) and tillers which emerged from the plants cut at harvest (here the number of plants with such tillers was counted).

Despite the security measures undertaken at harvest, such as harvesting individual plants by hand and collecting seeds from the soil surface, 1053 emergence events (i.e. individual seedlings, patches of seedlings and tillers) occurred in the 129.6 m^2 of field monitored during 6 months after harvest. Most of these emergence events (88.6%) came from individual seeds shed by plants. Due to low rates of after-harvest tiller development (5% of all the germination events), tillering does not appear to be an important mechanism of wheat persistence in the field. The emergence of patches of seedlings from the spikes also occurred rarely, only in 6.4% of all the germination

events. The patches of seedlings, however, had a higher survival rate (82.1%) than individual seedlings (56.3%) or tillers (52.8%), i.e. when a dense group of seedlings emerged there was a high probability that at least some plants from this group persisted for a longer period of time.

Although more individual seedlings emerged in the plots previously sown with non-GM wheat lines, this effect could be a result of a 2–3 days earlier harvesting of transgenic plants due to biosafety considerations, and thus more advanced ripening stage and consequently higher seed loss for non-GM wheat lines by the time of harvest. Among all the individual seedlings used for continuous monitoring, 53.3% survived winter and were persisting in the field 6 months after emergence, in March 2009.

We found no indication that the individual seedlings of GM wheat lines could persist longer than the seedlings of their conventional counterparts. However, 140 transgenic wheat plants were found to survive winter and persist in the field until spring and 12 GM plants even formed spikes. Unfortunately, but necessarily for biosafety reasons, it was not possible to monitor these plants for a longer time period and to assess the next year rates of re-seeding from the wheat plants persisting in the field; they had to be destroyed before flower opening.

We observed strong varietal differences in volunteer seedling mortality: the plants originating from Swiss varieties were better adapted to low winter temperatures and had lower mortality rates than the plants of the Mexican wheat variety Bobwhite. Thus adaptation to local environmental conditions appeared to be a more important predictor of overwinter survival than the introduced transgenes. Several other studies have previously reported high variability in volunteer (self-sown) seedling emergence and persistence of GM or conventional varieties of spring wheat or other GM crops in the field depending on genotypic, environmental or production factors (Anderson and Soper 2003, Gruber *et al.* 2004, Harker *et al.* 2005a, De Corby *et al.* 2007). Although in our study there were no differences in persistence of the transgenic disease-resistant and conventional plants, some other types of transgenes, in particular the genes that confer resistance to herbicides, can be more likely to enhance GM wheat persistence in fallow fields. In Canada, for example, glyphosate-resistant wheat recruitment was observed in the field even 3 years after sowing and the recruitment rates strongly depended on agricultural practice, i.e. tillage and herbicide application (Harker *et al.* 2005a). Some studies also reported long-term persistence of herbicide-resistant oilseed rape in or outside agricultural habitats (D'Hertefeldt *et al.* 2008). Some of the new transgenes,

such as those modifying seed quality or increasing overall plant fitness, are expected to have a stronger effect on persistence of GM crops through increasing seed survival or plant survival and fecundity (Claessen *et al.* 2005). It is therefore difficult to predict potential persistence and overwinter survival of new transgenic wheat varieties which would have to be thoroughly assessed on a case-by-case basis.

Interestingly, we observed continuous wheat seedling emergence from September until November 2008 but not later, in spring 2009. Because we have only assessed seedling emergence until April 2009, we cannot exclude the possibility that wheat seeds would also germinate in the field later, when soil temperatures rise. The short life of wheat seeds in soil (see above discussion of seed persistence in soil in the laboratory), however, indicates that newly emerged seedlings would rather result from a re-seeding by volunteer plants than from the soil seed bank. Although some studies reported the emergence of the seedlings of GM wheat in the field 16 months and even up to three years after harvest (Harker *et al.* 2005a, De Corby *et al.* 2007), these volunteering events were more likely a result of such re-seeding by volunteer wheat plants and depended on agricultural practice (Harker *et al.* 2005a). Our data support the point of view that persistence of escaped volunteer plants with subsequent re-seeding may be a more important persistence mechanism for spring wheat than the fast-decaying soil seed bank (De Corby *et al.* 2007).

Effects on post-harvest weed vegetation

The fourth question we asked was whether GM wheat cultivation might affect the structure of post-harvest weed communities. We found no effects of growing GM wheat on the diversity or structure of the fallow weed communities. Although it is known that some GM crops, in particular those with introduced herbicide resistance, can affect post-harvest vegetation (Harker *et al.* 2005b), these effects are largely caused by the agricultural practice, i.e. herbicide application, used with such crops and not directly by the transgenes. GM plants could also potentially impact vegetation indirectly via influencing soil microorganisms, non-target insect herbivores or persistence of the transgene product in soil (Dale *et al.* 2002, Gyamfi *et al.* 2002, Snow *et al.* 2003). In our case, however, the plants carrying *Pm3b* and *Chi* and *Glu* transgenes did not seem to change the post-harvest vegetation. Our findings correspond to the results of several companion studies which showed no effects of these particular GM plants on non-target organisms, such as insect species (Peter *et al.* 2010, von Burg *et al.*

2010, Alvarez-Alfageme *et al.* 2011), soil fauna (Duc *et al.* 2011) or soil beneficial bacteria (Song Wilson *et al.* 2010).

Application of soil fertilizer during the field season had no overall effect on species abundance in the fallow weed communities. Only one dominant species, *P. annua*, was found to be more abundant in previously fertilized plots in autumn. This confirms the results of some other studies which showed that grasses often benefit from fertilization and become dominant in plant communities in fertilized habitats at the expense of other species (Mountford *et al.* 1993, Foster and Gross 1998). We also observed a marginally significant effect of fertilization on the diversity of the weed communities (species richness) in autumn 2008: more species were found in unfertilized plots compared to the plots that were fertilized. This supports the theory that soil nitrification negatively affects species diversity (Mountford *et al.* 1993, Willems *et al.* 1993, Elisseou et al. 1995). Both these effects, however, disappeared by spring 2009, when fertilizer left in the soil after the field season had probably fully been taken up by plants or washed out from the soil.

Conclusions

The introduced transgenes conferring resistance to pathogenic fungi did not enhance the persistence of the wheat plants in weed communities commonly associated with wheat or on fallow plots compared to the corresponding conventional wheat lines. Rather, the higher expression of the resistance gene even incurred fitness costs in some GM lines when they had to withstand competition from weeds. Growing transgenic wheat lines had also no effect on the structure and diversity of the fallow weed communities. Our study shows, however, that GM wheat plants are able to persist and reproduce both among weeds and in the fallow over winter. Fast seed germination and short-term seed persistence in soil along with successful overwintering indicate that persistence of the seedlings and plants of transgenic wheat in fallow fields and subsequent re-seeding might be a more important mechanism of GM wheat persistence than germination from the soil seed bank. Strong varietal differences in persistence point out the importance of case-by-case assessment of new GM wheat varieties.

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Figures

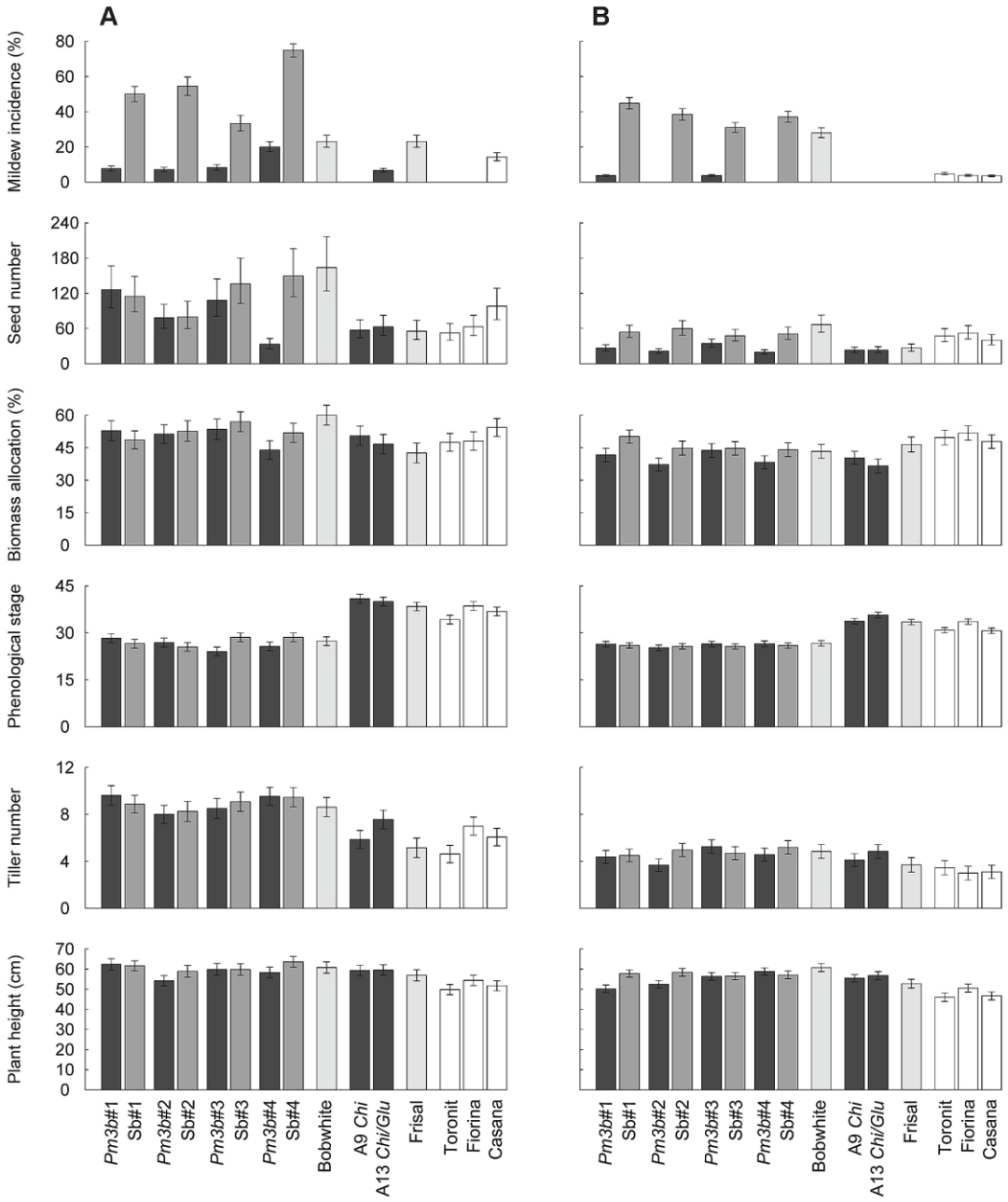


Figure 1. Mildew incidence and performance of the 15 wheat lines grown without competition (column A) and in the weed communities (column B). The data for high and low nutrient treatments and for two different weed communities are pooled. Bars represent means ± standard errors. Four grades of the grey scale indicate groups of wheat lines; from dark to light: transgenic lines, the genetically closest control (sister lines), wheat varieties used for transgene insertion and modern conventional wheat varieties.

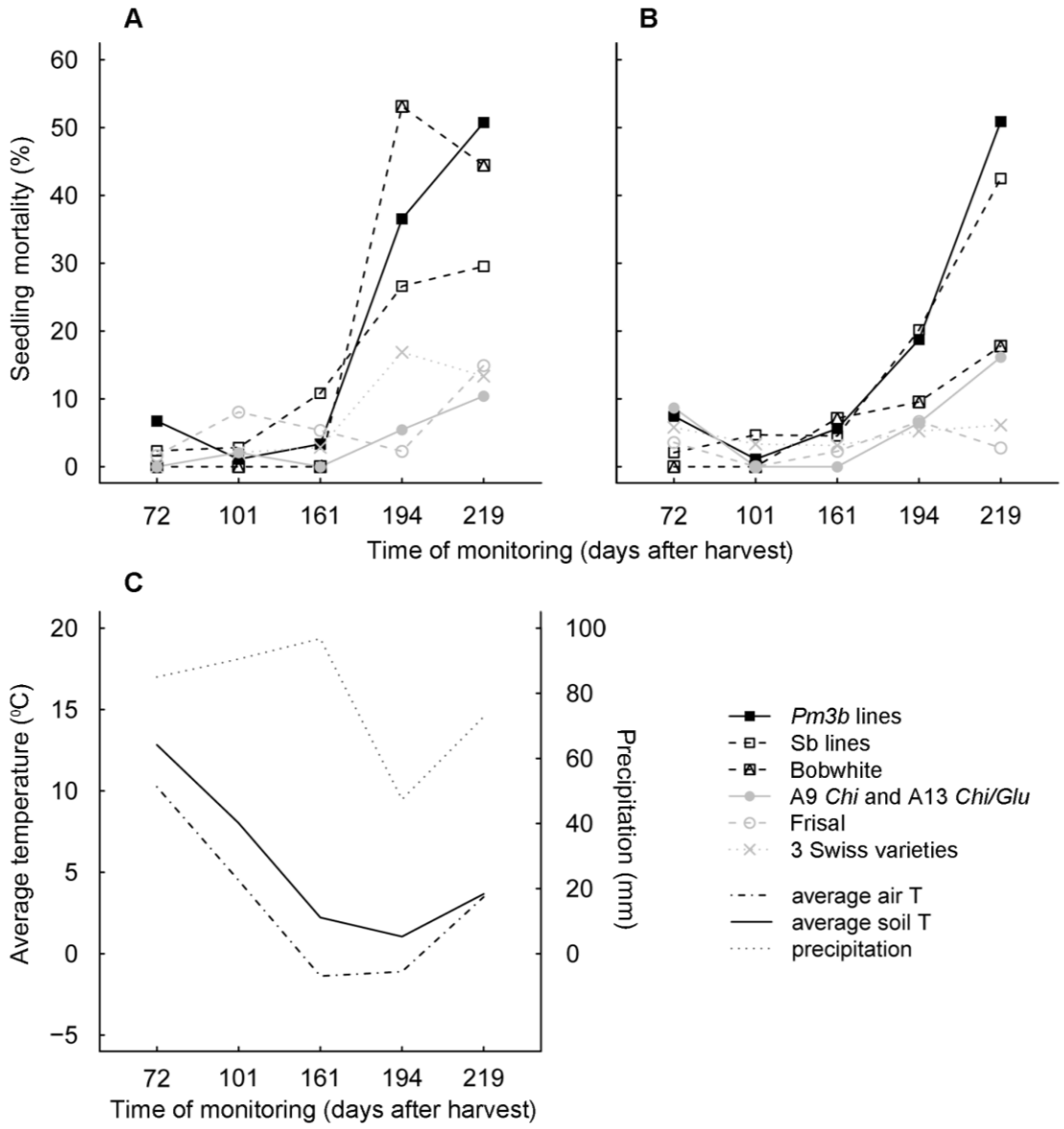


Figure 2. Mortality rates of seedlings of the GM and non-GM wheat lines and varieties (A, B) and weather conditions (C) from November 2008 until April 2009. Left chart (A) — no nutrient addition during vegetation season 2008. Right chart (B) — nutrient addition. Mortality rates in %, i.e. the percentage of dead seedlings out of total seedlings alive at previous count, are shown. Left chart (C) — weather conditions: average air and soil temperature measured in ^{0}C (left axis) and precipitation measure in mm (right axis). The meteorological data were provided by the Federal Office of Meteorology and Climatology of Switzerland.

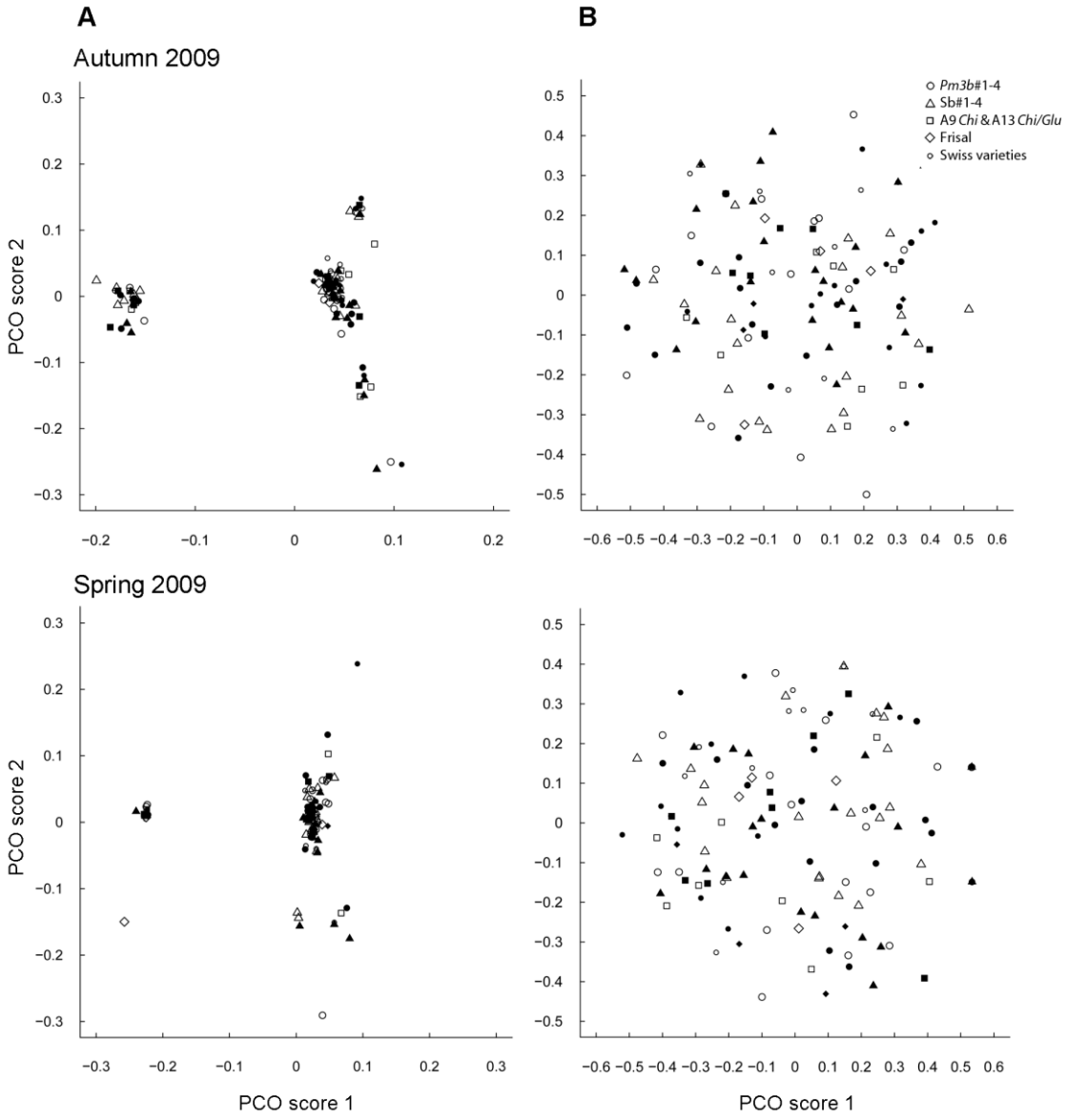


Figure 3. PCO ordinations for the abundance (column A, Euclidean distances matrix) and occurrence (column B, Jaccard similarity matrix) of the weed species in GM and non-GM wheat fallow plots. The axes are the first and the second ordination axes for principal coordinates analysis. The results of the two vegetation counts in autumn 2008 and in spring 2009 are presented. Open symbols indicate the plots which received no fertilizer, closed symbols those which were fertilized twice in the preceding field season 2008.

Supplemental Material to Chapter 2

Table S1. Weed species which were sown (in bold) or naturally occurring in weed mixture plots. Nomenclature follows Lauber and Wagner 1996.

Nr.	Weed mixture #1 species common for wheat fields	Weed mixture #2 species common for wheat fallow fields
1	***Apera spica-venti***	*Amaranthus albus*
2	*Amaranthus retroflexus*	***Amaranthus retroflexus***
3	***Anagallis arvensis***	*Anagallis arvensis*
4	***Arabidopsis thaliana***	*Asteracea* sp. 1 (unidentified)
5	*Asteracea* sp.1 (unidentified)	*Brassica napus*
6	*Asteracea* sp.2 (unidentified)	*Capsella bursa-pastoris*
7	*Brassica napus*	*Chenopodium album*
8	***Capsella bursa-pastoris***	***Echinochloa crusgalli***
9	***Centaurea cyanus***	*Euphorbia helioscopia*
10	*Convolvulus arvense*	***Fumaria officinalis***
11	***Dactylis glomerata***	***Galeopsis tetrahit***
12	*Echinochloa crus-galli*	*Galinsoga ciliata*
13	*Euphorbia helioscopia*	*Lamium amplexicaule*
14	*Galinsoga ciliata*	***Lamium purpureum***
15	*Lamium purpureum*	*Matricaria recutita*
16	***Lolium perenne***	***Plantago lanceolata***
17	*Matricaria recutita*	*Plantago major*
18	***Papaver rhoeas***	***Poa annua***
19	*Plantago lanceolata*	***Poa trivialis***
20	***Plantago major***	*Polygonum aviculare*
21	*Poa annua*	*Polygonum persicaria*
22	*Poa trivialis*	*Polygonum* sp. (unidentified)
23	***Polygonum aviculare***	***Raphanus raphanistrum***
24	*Polygonum persicaria*	*Raphanus raphanistrum*
25	*Polygonum* sp. (unidentified)	*Senecio vulgaris*
26	*Ranunculus acris*	*Solanum nigrum*
27	*Senecio vulgaris*	*Stellaria media*
28	*Solanum nigrum*	*Taraxacum officinale*
29	*Stellaria media*	***Trifolium pratense***
30	*Taraxacum officinale*	*Trifolium repens*
31	*Trifolium pratense*	*Verbascum thapsus*
32	***Trifolium repens***	*Veronica persica*
33	*Verbascum thapsus*	*Viola arvensis*
34	*Veronica persica*	
35	*Viola arvensis*	

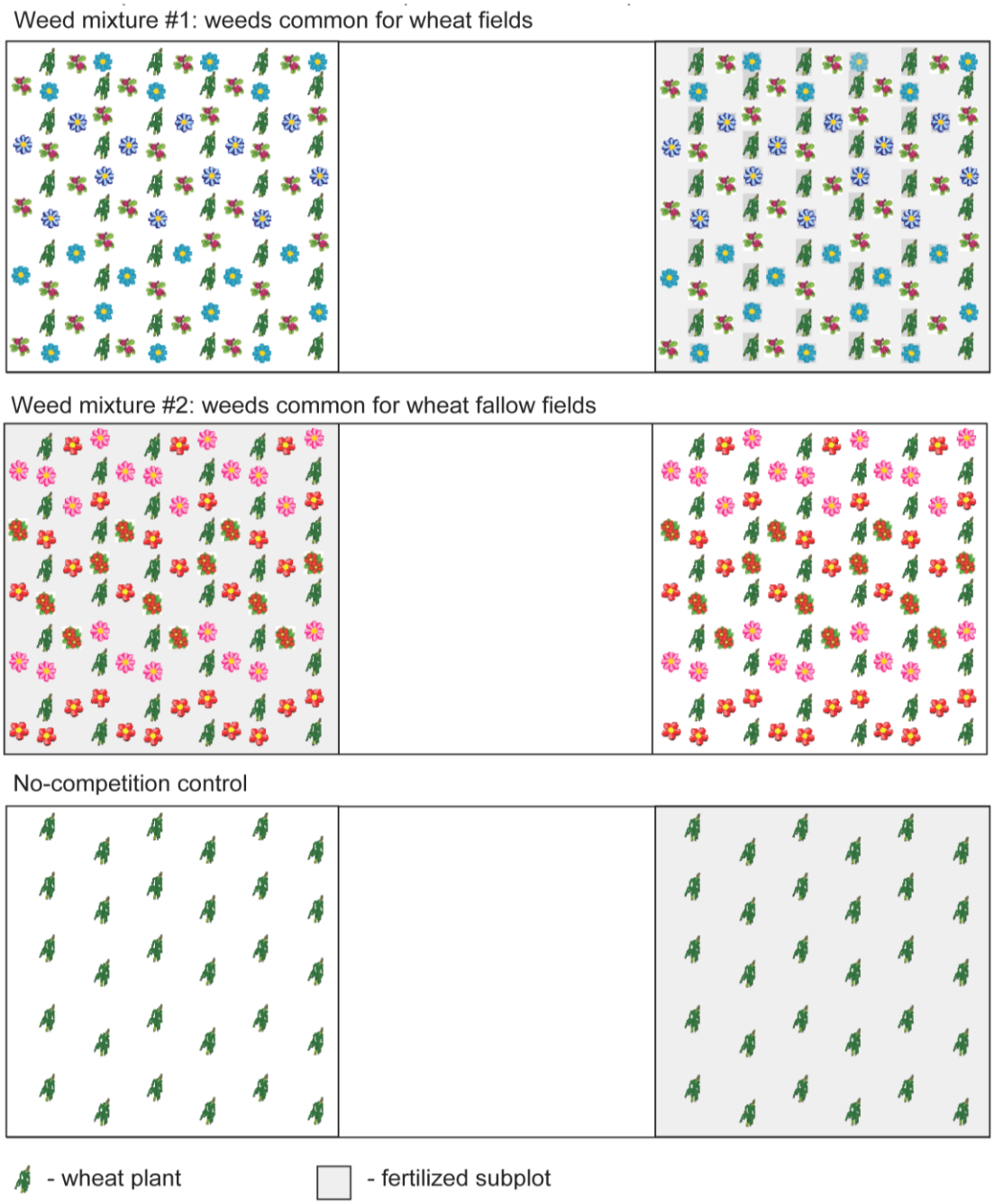
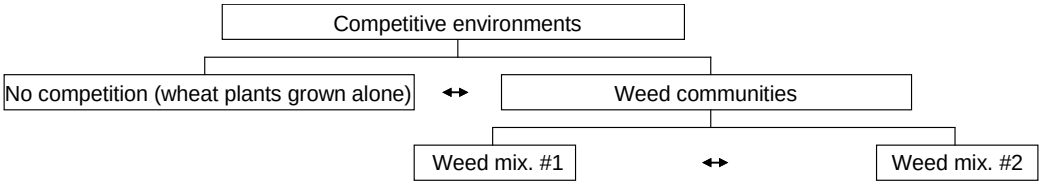


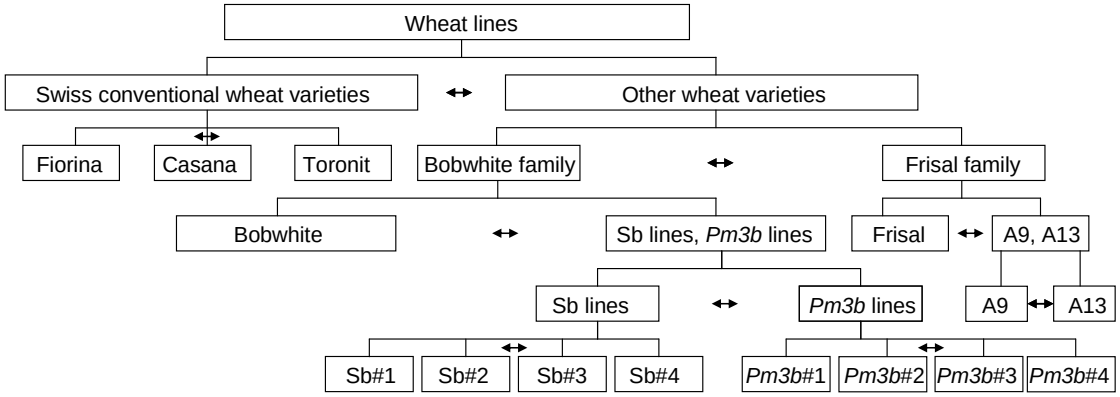
Figure S1. Scheme of the "weed" experiment. The scheme shows one of the four field blocks consisting of three plots in which either the wheat plants were planted into the two different weed communities or the wheat plants were grown alone, without competition. One of the 1×1.08 m subplots within each plot was treated with fertilizer (grey color), another was left untreated.

Figure S2. Photograph of the weed mixture plot with wheat plants.

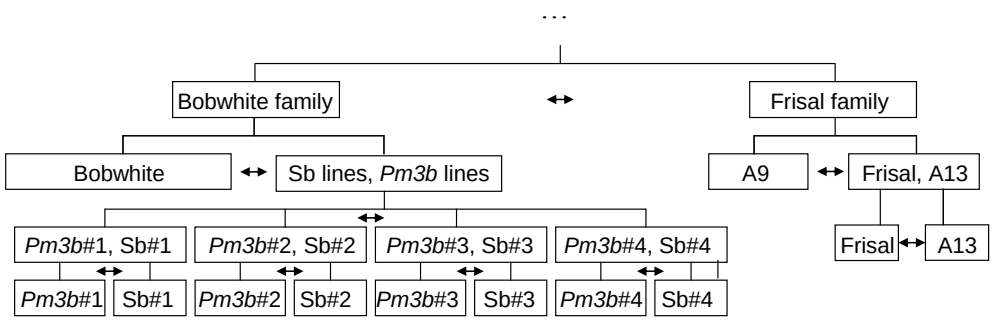


Three alternative models (comparisons of groups of GM and non-GM wheat lines or pairwise comparisons GM vs. Control):

Model 1.



Model 2.



Model 3.

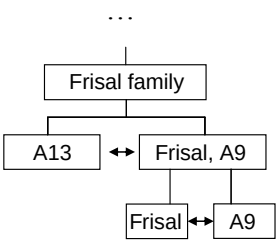


Figure S3. The structure of orthogonal contrasts used in the extended ANOVA models.

Table S2. ANOVA table showing effects of weed competition and fertilizer applied during the preceding field season on mildew incidence and seed number of 15 GM and non-GM wheat lines.

Source of variation	Mildew incidence (logit)			Seed number (log)		
	df	%SS	F pr.	df	%SS	F pr.
Basic model						
Block	3	0.20	0.630	3	1.88	0.055
Competitive environment	2	1.32	0.080	2	11.55	0.003
Plot	3	0.30	0.815	3	0.22	0.771
Fertilizer	1	3.82	0.013	1	1.68	0.025
Competitive environment×Fertilizer	2	0.79	0.352	2	0.80	0.204
Subplot	6	1.90	0.002	6	1.14	0.184
Wheat line	14	27.19	<.001	14	9.80	<.001
Competitive environment×Wheat line	28	5.97	0.112	28	4.55	0.669
Plot×Wheat line	84	12.59	0.001	83	15.72	0.008
Fertilizer×Wheat line	14	3.02	0.003	14	1.31	0.747
Residual	472	42.89		399	51.34	
Total	629	100.00		555	100.00	
Extended model						
Block	3	0.20	0.630	3	1.88	0.055
<u>Competitive environment contrasts:</u>						
No-competition vs. weed competition	1	1.17	0.042	1	11.52	0.001
Weed-mixture#1 vs. weed mixture#2	1	0.15	0.310	1	0.03	0.582
Plot	3	0.30	0.815	3	0.22	0.771
Fertilizer	1	3.82	0.013	1	1.68	0.025
Competitive environment×Fertilizer	2	0.79	0.352	2	0.80	0.204
Subplot	6	1.90	0.002	6	1.14	0.184
<u>Wheat line contrasts:</u>						
Swiss vs. other wheat	1	3.24	<.001	1	0.09	0.412
3 Swiss varieties	2	0.21	0.316	2	0.08	0.746
Bobwhite vs. Frisal	1	6.38	<.001	1	2.10	<.001
Bobwhite vs. *Pm3b* and Sb lines	1	0.04	0.518	1	1.25	0.002
Pm3b vs. Sb lines	1	15.32	<.001	1	4.51	<.001
Sb lines	3	0.75	0.043	3	0.03	0.975
Pm3b lines	3	0.25	0.435	3	1.72	0.004
A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.74	0.004	1	0.03	0.646
A9 *Chi* vs. A13 *Chi/Glu*	1	0.26	0.089	1	0.01	0.813
<u>Interactions:</u>						
Competitive environment×Wheat line	28	5.97	0.112	28	4.55	0.669
Plot×Wheat line	84	12.59	0.001	83	15.72	0.008
Fertilizer×Swiss vs. other wheat	1	0.12	0.242	1	0.04	0.566
Fertilizer×3 Swiss varieties	2	0.70	0.021	2	0.04	0.859
Fertilizer×Bobwhite vs. Frisal	1	0.01	0.734	1	0.003	0.880
Fertilizer×Bobwhite vs. *Pm3b* and Sb lines	1	0.00006	0.980	1	0.0001	0.975
Fertilizer×*Pm3b* vs. Sb lines	1	0.0002	0.962	1	0.24	0.176
Fertilizer×Sb lines	3	1.06	0.009	3	0.65	0.168
Fertilizer×*Pm3b* lines	3	1.11	0.007	3	0.27	0.548
Fertilizer×A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.02	0.648	1	0.06	0.483
Fertilizer×A9 *Chi* vs. A13 *Chi/Glu*	1	0.00006	0.980	1	0.001	0.925
Residual	472	42.89		399	51.34	
Total	629	100.00		555	100.00	

Table S3. ANOVA table showing effects of weed competition and fertilizer applied during the preceding field season on biomass allocation and tiller number of 15 GM and non-GM wheat lines.

Source of variation	Biomass allocation (%)			Tiller number		
	df	%SS	F pr.	df	%SS	F pr.
Basic model						
Block	3	7.87	0.173	3	2.09	0.236
Competitive environment	2	2.86	0.302	2	18.69	0.009
Plot	3	2.34	0.057	3	0.84	0.177
Fertilizer	1	0.01	0.867	1	8.67	<.001
Competitive environment×Fertilizer	2	0.11	0.739	2	2.92	0.008
Subplot	6	1.05	0.240	6	0.73	0.294
Wheat line	14	3.26	0.039	14	5.76	<.001
Competitive environment×Wheat line	28	5.34	0.449	28	3.08	0.545
Plot×Wheat line	84	15.64	0.013	84	9.72	0.164
Fertilizer×Wheat line	14	1.02	0.898	14	1.30	0.517
Residual	463	60.51		466	46.21	
Total	620	100.0		623	100.0	
Extended model						
Block	3	7.87	0.173	3	2.09	0.236
<u>Competitive environment contrasts:</u>						
No-competition vs. weed competition	1	2.84	0.152	1	18.59	0.004
Weed-mixture#1 vs. weed mixture#2	1	0.02	0.889	1	0.09	0.601
Plot	3	2.34	0.057	3	0.84	0.177
Fertilizer	1	0.01	0.867	1	8.67	<.001
Competitive environment×Fertilizer	2	0.11	0.739	2	2.92	0.008
Subplot	6	1.05	0.240	6	0.73	0.294
<u>Wheat line contrasts:</u>						
Swiss vs. other wheat	1	0.83	0.012	1	3.12	<.001
3 Swiss varieties	2	0.03	0.886	2	0.11	0.573
Bobwhite vs. Frisal	1	0.56	0.040	1	1.27	<.001
Bobwhite vs. *Pm3b* and Sb lines	1	0.12	0.348	1	0.002	0.851
Pm3b vs. Sb lines	1	0.81	0.013	1	0.13	0.254
Sb lines	3	0.13	0.811	3	0.12	0.752
Pm3b lines	3	0.63	0.188	3	0.45	0.208
A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.08	0.437	1	0.26	0.104
A9 *Chi* vs. A13 *Chi/Glu*	1	0.08	0.429	1	0.29	0.091
<u>Interactions:</u>						
Competitive environment×Wheat line	28	5.34	0.449	28	3.08	0.545
Plot×Wheat line	84	15.64	0.013	84	9.72	0.164
Fertilizer×Swiss vs. other wheat	1	0.24	0.179	1	0.09	0.343
Fertilizer×3 Swiss varieties	2	0.25	0.389	2	0.22	0.327
Fertilizer×Bobwhite vs. Frisal	1	0.08	0.422	1	0.04	0.527
Fertilizer×Bobwhite vs. *Pm3b* and Sb lines	1	0.01	0.744	1	0.02	0.638
Fertilizer×*Pm3b* vs. Sb lines	1	0.01	0.752	1	0.12	0.274
Fertilizer×Sb lines	3	0.06	0.930	3	0.35	0.324
Fertilizer×*Pm3b* lines	3	0.11	0.840	3	0.34	0.336
Fertilizer×A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.25	0.165	1	0.003	0.865
Fertilizer×A9 *Chi* vs. A13 *Chi/Glu*	1	0.003	0.873	1	0.13	0.262
Residual	463	60.51		466	46.21	
Total	620	100.0		623	100.0	

Table S4. ANOVA table showing effects of weed competition and fertilizer applied during the preceding field season on plant height and phenological stage of 15 GM and non-GM wheat lines.

Source of variation	Plant height (cm)			Phenological stage		
	df	%SS	F pr.	df	%SS	F pr.
Basic model						
Block	3	0.16	0.858	3	29.34	0.039
Competitive environment	2	1.83	0.136	2	2.84	0.335
Plot	3	0.66	0.652	3	2.64	0.073
Fertilizer	1	12.98	0.001	1	0.15	0.448
Competitive environment×Fertilizer	2	1.56	0.212	2	0.11	0.787
Subplot	6	2.29	0.004	6	1.35	0.017
Wheat line	14	10.27	<.001	14	14.41	<.001
Competitive environment×Wheat line	28	2.71	0.829	28	1.53	0.954
Plot×Wheat line	84	11.19	0.236	84	8.09	0.239
Fertilizer×Wheat line	14	1.46	0.582	14	0.94	0.693
Residual	461	54.88		448	38.60	
Total	618	100.00		605	100.00	
Extended model						
Block	3	0.16	0.858	3	29.34	0.039
Competitive environment contrasts:						
No-competition vs. weed competition	1	1.82	0.064	1	2.72	0.177
Weed-mixture#1 vs. weed mixture#2	1	0.01	0.851	1	0.11	0.742
Plot	3	0.66	0.652	3	2.64	0.073
Fertilizer	1	12.98	0.001	1	0.15	0.448
Competitive environment×Fertilizer	2	1.56	0.212	2	0.11	0.787
Subplot	6	2.29	0.004	6	1.35	0.017
Wheat line contrasts:						
Swiss vs. other wheat	1	6.90	<.001	1	3.79	<.001
3 Swiss varieties	2	0.47	0.141	2	0.29	0.187
Bobwhite vs. Frisal	1	0.12	0.317	1	9.39	<.001
Bobwhite vs. *Pm3b* and Sb lines	1	0.48	0.045	1	0.02	0.651
Pm3b vs. Sb lines	1	0.90	0.006	1	0.00001	0.991
Sb lines	3	0.08	0.878	3	0.16	0.614
Pm3b lines	3	1.08	0.030	3	0.73	0.039
A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.22	0.179	1	0.03	0.532
A9 *Chi* vs. A13 *Chi/Glu*	1	0.03	0.633	1	0.0005	0.940
Interactions:						
Competitive environment×Wheat line	28	2.71	0.829	28	1.53	0.954
Plot×Wheat line	84	11.19	0.236	84	8.09	0.239
Fertilizer×Swiss vs. other wheat	1	0.08	0.403	1	0.002	0.894
Fertilizer×3 Swiss varieties	2	0.54	0.107	2	0.18	0.351
Fertilizer×Bobwhite vs. Frisal	1	0.05	0.510	1	0.03	0.549
Fertilizer×Bobwhite vs. *Pm3b* and Sb lines	1	0.02	0.649	1	0.05	0.464
Fertilizer×*Pm3b* vs. Sb lines	1	0.14	0.284	1	0.12	0.240
Fertilizer×Sb lines	3	0.09	0.860	3	0.10	0.752
Fertilizer×*Pm3b* lines	3	0.23	0.581	3	0.20	0.511
Fertilizer×A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.20	0.199	1	0.11	0.249
Fertilizer×A9 *Chi* vs. A13 *Chi/Glu*	1	0.11	0.332	1	0.14	0.200
Residual	461	54.88		448	38.60	
Total	618	100.00		605	100.00	

Table S5. ANOVA table showing effects of time, oxygen availability, soil humidity and fertilizer obtained by the mother plant on the percentage of seeds of 15 wheat lines germinated during storage in the climate chamber.

Source of variation	df	%SS	F pr.
Basic model			
Replicate	4	0.71	<.001
Time (3 months vs. 6 months of storage)	1	5.51	<.001
Oxygen (aerobic vs. anaerobic conditions)	1	36.19	<.001
Soil humidity	1	12.13	<.001
Fertilizer	1	0.04	0.34
Wheat line	14	1	0.02
Time×Wheat line	14	0.19	0.986
Oxygen×Wheat line	14	0.81	0.095
Soil humidity×Wheat line	14	0.5	0.515
Fertilizer×Wheat line	14	0.31	0.885
Residual	1121	42.59	
Total	1199	100	
Extended model			
Replicate	4	0.71	<.001
Time (3 months vs. 6 months of storage)	1	5.51	<.001
Oxygen (aerobic vs. anaerobic conditions)	1	36.2	<.001
Time×Oxygen	1	2.96	<.001
Soil humidity	1	12.1	<.001
Soil humidity×Time	1	0.63	<.001
Soil humidity×Oxygen	1	15.5	<.001
Fertilizer	1	0.04	0.199
Time×Fertilizer	1	0.13	0.014
Oxygen×Fertilizer	1	0.0004	0.892
Soil humidity×Fertilizer	1	0.04	0.187
Wheat line contrasts:			
Swiss vs. other wheat	1	0.03	0.211
3 Swiss varieties	2	0.38	<.001
Bobwhite vs. Frisal	1	0.01	0.407
Bobwhite vs. *Pm3b* and Sb lines	1	0.38	<.001
Pm3b vs. Sb lines	1	0.004	0.643
Pm3b lines	3	0.12	0.117
Sb lines	3	0.04	0.648
Frisal vs. A9 *Chi* and A13 *Chi/Glu*	1	0.06	0.097
A9 *Chi* vs. A13 *Chi/Glu*	1	0.002	0.781
Interactions:			
Time×Swiss vs. other wheat	1	0.004	0.66
Time×3 Swiss varieties	2	0.0002	0.994
Time×Bobwhite vs. Frisal	1	0.02	0.315
Time×Bobwhite vs. *Pm3b* and Sb lines	1	0.05	0.134
Time×*Pm3b* vs. Sb lines	1	0.02	0.404
Time×Sb lines	3	0.03	0.756
Time×*Pm3b* lines	3	0.06	0.455
Time×Frisal vs. A9 *Chi* and A13 *Chi/Glu*	1	0.02	0.363
Time×A9 *Chi* vs. A13 *Chi/Glu*	1	0.004	0.643
Oxygen×Swiss vs. other wheat	1	0.16	0.006
Oxygen×3 Swiss varieties	2	0.18	0.013
Oxygen×Bobwhite vs. Frisal	1	0.08	0.052
Oxygen×Bobwhite vs. *Pm3b* and Sb lines	1	0.16	0.005
Oxygen×*Pm3b* vs. Sb lines	1	0.05	0.138
Oxygen×Sb lines	3	0.1	0.183
Oxygen×*Pm3b* lines	3	0.02	0.774

Table S5 continues

Source of variation	df	%SS	F pr.
Oxygen×Frisal vs. A9 *Chi* and A13 *Chi/Glu*	1	0.05	0.149
Oxygen×A9 *Chi* vs. A13*Chi/Glu*	1	0.01	0.517
Soil humidity×Swiss vs. other wheat	1	0.07	0.073
Soil humidity×3 Swiss varieties	2	0.04	0.355
Soil humidity×Bobwhite vs. Frisal	1	0.08	0.05
Soil humidity×Bobwhite vs. *Pm3b* and Sb lines	1	0.11	0.021
Soil humidity×*Pm3b* vs. Sb lines	1	0.04	0.151
Soil humidity×Sb lines	3	0.08	0.256
Soil humidity×*Pm3b* lines	3	0.05	0.483
Soil humidity×Frisal vs. A9 *Chi* and A13 *Chi/Glu*	1	0.004	0.669
Soil humidity×A9 *Chi* vs. A13 *Chi/Glu*	1	0.01	0.459
Fertilizer×Swiss vs. other wheat	1	0.006	0.588
Fertilizer×3 Swiss varieties	2	0.002	0.961
Fertilizer×Bobwhite vs. Frisal	1	0.02	0.382
Fertilizer×Bobwhite vs. *Pm3b* and Sb lines	1	0.02	0.338
Fertilizer×*Pm3b* vs. Sb lines	1	0.00005	0.963
Fertilizer×Sb lines	3	0.05	0.475
Fertilizer×*Pm3b* lines	3	0.19	0.031
Fertilizer×Frisal vs. A9 *Chi* and A13 *Chi/Glu*	1	0.003	0.708
Fertilizer×A9 *Chi* vs. A13 *Chi/Glu*	1	0.02	0.308
Residual	1115	23.4	
Total	1199	100	

Table S6. The ANOVA table (GLLM) shows the effects of time between monitoring counts, time of count, the wheat line (genotype) and fertilizer on seedling mortality rates of 15 wheat lines in the field from autumn 2008 – spring 2009.

Source of variation	df	F pr.
Log (days between counts)	1	<0.001
Time of count (count events)	3	<0.001
Wheat line contrasts:		
Swiss vs. other wheat	1	0.002
3 Swiss varieties	2	0.59
Bobwhite vs. Frisal	1	<0.001
Bobwhite vs. *Pm3b* and Sb lines	1	0.791
Pm3b vs. Sb lines	1	0.279
Pm3b lines	3	0.104
Sb lines	3	0.544
A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.537
A9 *Chi* vs. A13 *Chi/Glu*	1	0.496
Interactions:		
Log (days between counts)×Swiss vs. other wheat	1	0.004
Time of count×Swiss vs. other wheat	3	0.209
Log (days between counts)×3 Swiss varieties	2	0.019
Time of count×3 Swiss varieties	6	0.997
Log (days between counts)×Bobwhite vs. Frisal	1	0.01
Time of count×Bobwhite vs. Frisal	3	0.224
Log (days between counts)×Bobwhite vs. *Pm3b* and Sb lines	1	0.567
Time of count×Bobwhite vs. *Pm3b* and Sb lines	3	0.491
Log (days between counts)×*Pm3b* vs. Sb lines	1	0.6
Time of count×*Pm3b* lines vs. Sb lines	3	0.367
Log (days between counts)×*Pm3b* lines	3	0.12
Time of count×*Pm3b* lines	9	0.609
Log (days between counts)×Sb lines	3	0.934
Time of count×Sb lines	9	0.517
Log (days between counts)×A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.936
Time of count×A9 *Chi* and A13 *Chi/Glu* vs. Frisal	3	0.886
Log (days between counts)×A9 *Chi* vs. A13 *Chi/Glu*	1	0.607
Time of count×A9 *Chi* vs. A13 *Chi/Glu*	3	0.995
Fertilizer	1	0.05
Interactions:		
Log (days between counts)×Fertilizer	1	0.073
Time of count×Fertilizer	3	0.422
Swiss vs. other wheat×Fertilizer	1	0.934
3 Swiss varieties×Fertilizer	2	0.434
Bobwhite vs. Frisal× Fertilizer	1	0.5
Bobwhite vs. *Pm3b* and Sb lines×Fertilizer	1	0.151
Pm3b vs. Sb lines×Fertilizer	1	0.517
Pm3b lines×Fertilizer	3	0.996
Sb lines×Fertilizer	3	0.033
A9 *Chi* and A13 *Chi/Glu* vs. Frisal×Fertilizer	1	0.068
A9 *Chi* vs. A13 *Chi/Glu*×Fertilizer	1	0.687

Table S7. Weed species occurring in the plots previously sown with wheat at two vegetation surveys in November 2008 and in April 2009. Nomenclature follows Lauber and Wagner 1996.

Species	November 2008	April 2009
Apera spica-venti	–	+
Amaranthus albus	+	–
Amaranthus retroflexus	+	–
Anagallis arvensis	+	+
Asteracea sp. (unidentified)	+	–
Brassica napus	+	+
Capsella bursa-pastoris	+	+
Centaurea cyanus	–	+
Cerastium fontanum	+	–
Chenopodium album	+	–
Chenopodium polyspermum	+	–
Convolvulus arvensis	+	+
Crepis tectorum	+	–
Echinochloa cruss-galli	+	+
Epilobium tetragonum	+	–
Euphorbia cyparissias	+	+
Euphorbia maculata	+	–
Fumaria officinalis	+	–
Galeopsis tetrahit	+	–
Galinsoga parviflora	+	–
Geranium pusilum	+	+
Lamium purpureum	+	+
Linaria vulgaris	+	+
Lolium perenne	+	+
Matricaria recutita	+	+
Medicago lupulina	+	+
Oxalis acetosella	+	–
Papaver rhoeas	–	+
Phacelia tanacetifolia	+	–
Plantago lanceolata	+	+
Plantago major	+	+
Plantago media	+	+
Poa annua	+	+
Poa trivialis	+	+
Polygonum avicularis	+	+
Polygonum persicum	+	–
Primula elatior	–	+
Ranunculus acris	+	–
Raphanis raphanistrum	+	–
Rosa canina	+	–
Scorzonera humilis	+	–
Seneceo vulgaris	+	+
Setaria viridis	+	–
Solanum nigrum	+	–
Stellaria media	–	+
Taraxacum officinale	+	+
Tragopogon pratensis	+	+
Trifolium pratensis	+	+
Trifolium repens	+	+
Verbascum thapsus	+	+
Veronica persica	+	+
Viola arvensis	+	+

Table S8. ANOVA table showing effects of wheat line and fertilizer applied during in preceding field season on species richness of the post-harvest weed communities.

Source of variation	df	November 2008		April 2009	
		%SS	F pr.	%SS	F pr.
Block	3	14.01	0.001	3.15	0.335
Wheat line contrasts:					
Swiss vs. other wheat	1	0.81	0.303	2.03	0.141
3 Swiss varieties	2	2.10	0.253	1.65	0.408
Bobwhite vs. Frisal	1	0.01	0.893	2.95	0.078
Bobwhite vs. *Pm3b* and Sb lines	1	3.48	0.036	2.17	0.128
Pm3b vs. Sb lines	1	0.46	0.436	2.44	0.107
Pm3b lines	3	4.14	0.151	4.23	0.212
Sb lines	3	6.11	0.054	0.30	0.952
A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.08	0.743	0.14	0.694
A9 *Chi* vs. A13 *Chi/Glu*	1	2.18	0.094	1.37	0.224
Plot	43	31.89	0.151	38.78	0.145
Fertilizer	1	1.97	0.063	0.19	0.596
Interactions:					
Swiss vs. other wheat×Fertilizer	1	1.57	0.095	3.55	0.024
3 Swiss varieties×Fertilizer	2	0.25	0.795	0.54	0.665
Bobwhite vs. Frisal×Fertilizer	1	0.05	0.754	3.85	0.019
Bobwhite vs. *Pm3b* and Sb lines×Fertilizer	1	1.56	0.096	0.03	0.831
Pm3b vs. Sb lines×Fertilizer	1	1.09	0.162	0.61	0.339
Pm3b lines×Fertilizer	3	3.11	0.141	0.85	0.731
Sb lines×Fertilizer	3	1.24	0.520	1.12	0.638
A9 *Chi* and A13 *Chi/Glu* vs. Frisal×Fertilizer	1	0.02	0.848	0.46	0.407
A9 *Chi* vs. A13 *Chi/Glu*×Fertilizer	1	0.06	0.740	0.83	0.266
Residual	44	23.80		28.75	
Total	119	100.00		100.00	

Table S9. ANOVA table showing effects of wheat line and fertilizer applied during the preceding field season on Shannon-Weiner diversity index of the post-harvest weed communities.

Source of variation	df	November 2008		April 2009	
		%SS	F pr.	%SS	F pr.
Block	3	3.80	0.283	0.96	0.830
Wheat line contrasts:					
Swiss vs. other wheat	1	1.20	0.270	2.11	0.172
3 Swiss varieties	2	1.63	0.437	1.43	0.525
Bobwhite vs. Frisal	1	0.07	0.786	0.46	0.521
Bobwhite vs. *Pm3b* and Sb lines	1	0.22	0.639	2.55	0.134
Pm3b vs. Sb lines	1	1.43	0.231	1.07	0.328
Pm3b lines	3	2.87	0.406	0.34	0.957
Sb lines	3	8.08	0.052	7.26	0.101
A9 *Chi* and A13 *Chi/Glu* vs. Frisal	1	0.34	0.558	0.20	0.667
A9 *Chi* vs. A13 *Chi/Glu*	1	1.23	0.266	0.46	0.521
Plot	43	41.53	0.192	47.06	0.022
Fertilizer	1	0.95	0.263	0.74	0.268
Interactions:					
Swiss vs. other wheat×Fertilizer	1	0.42	0.455	2.38	0.050
3 Swiss varieties×Fertilizer	2	0.01	0.991	0.19	0.851
Bobwhite vs. Frisal×Fertilizer	1	0.90	0.277	0.22	0.547
Bobwhite vs. *Pm3b* and Sb lines×Fertilizer	1	0.70	0.335	0.34	0.448
Pm3b vs. Sb lines×Fertilizer	1	0.12	0.694	0.40	0.415
Pm3b lines×Fertilizer	3	0.56	0.859	1.06	0.617
Sb lines×Fertilizer	3	0.50	0.879	5.45	0.036
A9 *Chi* and A13 *Chi/Glu* vs. Frisal×Fertilizer	1	0.46	0.433	0.05	0.765
A9 *Chi* vs. A13 *Chi/Glu*×Fertilizer	1	0.38	0.477	0.08	0.721
Residual	44	32.59		25.18	
Total	119	100.00		100.00	

Table S10. ANOVA table showing effects of wheat line and fertilizer applied during the preceding field season on total canopy cover of the post-harvest weed communities.

Source of variation	df	November 2008		April 2009	
		%SS	F pr.	%SS	F pr.
Block	3	2.21	0.638	1.91	0.577
Wheat line contrasts:					
Swiss vs. other wheat	1	4.66	0.064	0.30	0.578
3 Swiss varieties	2	0.12	0.956	0.74	0.680
Bobwhite vs. Frisal	1	4.40	0.072	0.18	0.664
Bobwhite vs. *Pm3b* and Sb lines	1	1.98	0.222	0.33	0.558
Pm3b vs. Sb lines	1	0.29	0.640	0.08	0.773
Pm3b lines	3	3.06	0.506	0.59	0.891
Sb lines	3	0.50	0.942	0.35	0.946
Frisal vs. A9 *Chi* and A13 *Chi/Glu*	1	0.35	0.605	0.17	0.678
A9 *Chi* vs. A13 *Chi/Glu*	1	0.09	0.798	0.02	0.886
Plot	43	55.47	0.001	41.09	0.536
Fertilizer	1	0.13	0.606	1.28	0.259
Interactions:					
Swiss vs. other wheat×Fertilizer	1	0.43	0.358	0.75	0.388
3 Swiss varieties×Fertilizer	2	0.10	0.908	1.01	0.601
Bobwhite vs. Frisal×Fertilizer	1	0.01	0.876	1.23	0.269
Bobwhite vs. *Pm3b* and Sb lines×Fertilizer	1	0.97	0.170	1.06	0.305
Pm3b vs. Sb lines×Fertilizer	1	0.29	0.453	2.89	0.093
Pm3b lines × Fertilizer	3	2.45	0.193	2.52	0.471
Sb lines × Fertilizer	3	0.37	0.861	0.11	0.990
A9 *Chi* and A13 *Chi/Glu* vs. Frisal × Fertilizer	1	0.18	0.552	0.03	0.870
A9 *Chi* vs. A13 *Chi/Glu* × Fertilizer	1	0.09	0.680	0.08	0.776
Residual	44	21.88		43.26	
Total	119	100.00		100.00	

CHAPTER 3

Transgene × Environment Interactions in Genetically Modified Wheat

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Experiments with wheat lines in the glasshouse (mildew infection on the leaves) and in the field

Abstract

The introduction of transgenes into plants may cause unintended phenotypic effects which could have an impact on the plant itself and the environment. Little is published in the scientific literature about the interrelation of environmental factors and possible unintended effects in genetically modified (GM) plants.

We studied transgenic bread wheat *Triticum aestivum* lines expressing the wheat *Pm3b* gene against the fungus powdery mildew *Blumeria graminis* f.sp. *tritici*. Four independent offspring pairs, each consisting of a GM line and its corresponding non-GM control line, were grown under different soil nutrient conditions and with and without fungicide treatment in the glasshouse. Furthermore, we performed a field experiment with a similar design to validate our glasshouse results.

The transgene increased the resistance to powdery mildew in all environments. However, GM plants reacted sensitive to fungicide spraying in the glasshouse. Without fungicide treatment, in the glasshouse GM lines had increased vegetative biomass and seed number and a twofold yield compared with control lines. In the field these results were reversed. Fertilization generally increased GM/control differences in the glasshouse but not in the field.

Two of four GM lines showed up to 56% yield reduction and a 40-fold increase of infection with ergot disease *Claviceps purpurea* compared with their control lines in the field experiment; one GM line was very similar to its control.

Our results demonstrate that, depending on the insertion event, a particular transgene can have large effects on the entire phenotype of a plant and that these effects can sometimes be reversed when plants are moved from the glasshouse to the field. However, it remains unclear which mechanisms underlie these effects and how they may affect concepts in molecular plant breeding and plant evolutionary ecology.

Introduction

The widespread use of genetically modified (GM) plants in agriculture, together with the growing number of different crop species and introduced genes, demands sound environmental risk assessment (Wolfenbarger and Phifer 2000, Conner *et al.* 2003, Cellini *et al.* 2004, Snow *et al.* 2005). Following a tiered approach (Hill and Sendashonga 2003), data from such preliminary risk assessment usually form the basis for extended field trials or lead to the rejection of GM plants from further testing at an early stage (Conner and Christey 1994). Such studies often focus on the risk that a transgene may not show the desired phenotypic effect if the GM plants are moved from the controlled glasshouse environment to the more variable field conditions. However, few studies have reported potentially unintended phenotypic effects of transgenes in GM plants exposed to a range of realistic environmental conditions (Purrington and Bergelson 1995, Gertz *et al.* 1999). From evolutionary and ecological studies on wild plants it is well known that genotype × environment interactions can be large (Schlichting 1986, Sultan 1987, Schmid 1992, Sultan 2001, Yahiaoui *et al.* 2004), suggesting that similar interactions might occur in GM plants exposed to different environments, including glasshouse versus field environments. Plant breeders know intuitively that plant performance needs to be tested in realistic agricultural environments and regulatory authorities demand such assessments in their guidelines (EFSA 2006). Recent studies compared metabolic composition and transcriptional changes in GM Maize grown among environments and *in vitro* and outdoors (Coll *et al.* 2009, Barros *et al.* 2010). They found that differences between GM and control plants in metabolic profiles observed under standardized laboratory conditions were lost in the field. However, whether the same was true for ecological traits was not reported in these studies. Furthermore, a careful search in the literature for replicated and randomized studies about the ecological behaviour of GM and control plants in glasshouse versus field environments did not return any published references.

We therefore used the spring wheat variety Bobwhite SH 98 26 *Triticum aestivum* L. — transformed with the wheat *Pm3b* powdery mildew resistance gene (Yahiaoui *et al.* 2004) — as a model system to study potential transgene × environment interactions in genetically modified plants. We grew four offspring pairs, each consisting of a GM line and its corresponding non-GM control line under different soil nutrient conditions and fungicide treatment in the glasshouse and the field. Although well studied and not showing any abnormalities in the glasshouse, these plants had

never been planted outdoors prior to our experiments. We investigated to what extent the single inserted transgene could influence the disease resistance and overall fitness of our study plants and how these effects were modified by moving the plants from the glasshouse to the field. Since the germination rate of our plants was close to 100% (S. Zeller, unpublished data), agronomical performance traits such as seed yield and seed number were used to indirectly assess changes in plant fitness (Haldane 1927). We asked the following questions: (i) Does the transgene enhance resistance to powdery mildew *Blumeria graminis* f.sp. *tritici* (DC.) Speer and does it have other phenotypic effects such as fitness costs? (ii) Do we find these effects in all transformed lines or is there line-specific variation? (iii) Can intended and unintended effects of the transgene be influenced by environmental factors and are such effects detectable both in the glasshouse and in the field? We consider this study both as an example of how the ecological behaviour of genetically modified plants can be studied with experimental approaches and how such research can lead to insights into phenotypic effects of inserting a single gene artificially into a plant.

Materials and Methods

Genetically modified wheat

We used four wheat lines carrying the transgene *Pm3b* in different position on the genome and their respective non-transgenic control lines (null-segregants), each derived from different transformation events (von Burg *et al.* 2010, Peter *et al.* 2010). *Pm3b* confers race-specific resistance to powdery mildew and was cloned from hexaploid wheat (Yahiaoui *et al.* 2004). The lines were generated by biolistic transformation of spring wheat variety Bobwhite SH 98 26 (Pellegrineschi *et al.* 2002). The plasmids pAHC17+NotI (*PMI*) and pAHC17+3NotI (*Pm3b*) were used as vectors (Christensen and Quail 1996, Travella *et al.* 2006). After *Not*I (for *Pm3b*) or *Not*I/*Hin*dIII (for *PMI*) digestion, only the desired fragments, but no vector sequences, were co-bombarded into wheat. The *Pm3b* gene was cloned under the control of the *Zea mays* L. (maize) ubiquitin promoter (Christensen and Quail 1996) and transformants were selected on mannose-containing media using the phosphomannose isomerase (PMI)-coding gene as selectable marker (Reed *et al.* 2001). After regeneration of T_0 transformants, four independent T_1 families were selected. From each T_1 family, an offspring pair was further propagated consisting of a homozygous transgenic plant (GM lines *Pm3b*#1–4) and a null-segregant, i.e. a plant that did neither inherit the *Pm3b* transgene nor the

selectable marker (control lines Sb#1–4). Absence/presence of the transgenes was confirmed by Southern hybridization analysis (Southern 2006) using probes from the PM3B (bp 1231–1956 as referred to the GenBank accession AY325736) and PMI (bp 271–810 as referred to the GenBank accession AAC74685) encoding region. The GM lines contained the *Pmi* gene as well as one complete copy of *Pm3b*, and in the case of *Pm3b*#4 an additional fragment, which segregated as a single Mendelian locus in the T_1 generation. The null-segregants did not show any hybridization signal with the probes from the *Pm3b* as well as the *Pmi* coding genes. For both transgenic as well as null-segregant lines we can not exclude the presence of fragments from the coding genes or promoter/terminator regions which were not covered by the probes used in Southern blotting. The offspring pairs were multiplied to T_4 and used for the glasshouse and field experiments. The seeds used in this study were thus obtained from GM and control lines that had passed through four generations of sexual reproduction. Studies with *Drosophila melanogaster* (Henikoff 1979) and *Saccharomyces cerevisiae* (Gottschling *et al.* 1990) showed that a gene's position on the chromosome can influence its expression. We therefore assessed the expression level of the *Pm3b* transgene in the four GM lines by semi-quantitative RT-PCR using RNA isolated from leaves of seedlings grown in the glasshouse (Figure S1). As control for equal amount and quality of template cDNA, the expression levels of the *Mlo* gene (Yu *et al.* 2005) were determined.

Glasshouse experiment

The glasshouse experiment took place in a climate-controlled glasshouse at the Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Switzerland, from August 2007 to February 2008 (day/night temperature: 21/16 C°; additional light: 14 h/10 h day/night period, daily watering by hand). Seedlings of each line were planted individually into 11 cm square pots containing sterilized soil (Ökohum lawn soil, Ökohum AG, Herrenhof, Switzerland). The design consisted of the four GM and the four control wheat lines crossed with three soil nutrient levels (0, 1 or 2 g of "Osmocote exact mini" per L; Scotts, Waardenburg, The Netherlands). One gram of Osmocote per L corresponded to 13.2 g N, 6.6 g P, 9.1 g K and 1.7 g Mg m^{-2}. Natural infection of the wheat plants by powdery mildew occurred 1 month after planting. One half of the experiment was subsequently sprayed with a systemic fungicide specific to mildew (2 ml l^{-1} Opus Top; 83.7 g l^{-1} Epoxiconazol and 250 g l^{-1} Fenpropionazol; Maag

Agro AG, Dielsdorf, Switzerland). The active ingredient epoxiconazol blocks fungal cell pathways and activates the plants pathogen defences whereas fenpropionazol blocks two enzymes that are related to the fungal cell-wall synthesis. We used a high fungicide concentration (2ml/l); this caused slight leaf chlorosis on several plants that disappeared after a few days. All tested lines were affected equally. Each of the 8 × 3 line-by-nutrient level combinations was replicated five times. Plants were harvested 162 days after the start of the experiment.

Field experiment

The field experiment took place at an agricultural research station in Zurich-Reckenholz, Switzerland. It started in March 2008 and lasted until August 2008. Four replicate blocks, each with sixteen 1 × 1.08 m plots, were sown with seeds of the same eight wheat lines as used in the glasshouse experiment. In each plot, 400 seeds were sown in six rows with a distance of 18 cm between rows using an Oyjord plot drill system (Wintersteiger AG, Ried, Austria). Fertilizer was applied at the phenological stage 11 and 39 (Zadoks *et al.* 1974) to half of the plots (two times 3 g N m^{-2} as "Ammonsalpeter 27.5", Lonza, Visp, Switzerland).

The natural field soil provided the plants with sufficient phosphorous, potassium and magnesium (80, 235 and 234 mg kg^{-1}). All plots were sprayed with the herbicide cocktail Concert SX (40% Thifensulfurone, 4% Metusulfurone-methyl; Stähler Suisse AG, Zofingen, Switzerland) and Starane super (120 g l^{-1} Bromoxynil, 120 g l^{-1} Ioxynil, 100 g l^{-1} Fluroxypyr-metilheptil-ester; Omya Agro AG, Safenwil, Switzerland) in the beginning of May. In each plot, five individual plants were marked shortly after germination. Powdery mildew and ergot *Claviceps purpurea* (FR.) TUL. infection occurred naturally. Vandals damaged 53 of the 64 plots at random by removing the tops of some plants early in the flowering stage. The damage-induced loss of leaf area was within the natural variation observed in the field and smaller than the herbivory caused by *Oulema melanopus* L. (cereal leaf beetle). The damaged plots recovered within 2–3 weeks and regained their original height and vegetative mass. We recorded the exact area of damage within each plot and replaced all marked plants that had suffered damage (46.3%). A second field experiment with the same plant lines was carried out in an adjacent field the following year. Although plants grew higher because of more favourable weather conditions, the different wheat lines performed very similar as in

the 2008 trials (S. Zeller *et al.*, unpublished data). We are therefore confident that the here presented results and conclusions were not influenced by this disturbance.

Response Variables

We assessed the degree of powdery mildew infection (Eyal *et al.* 1987) and the phenological stage (Zadoks *et al.* 1974) 80 days after planting. Plants with visible powdery mildew colonies on all their leaves (including flag leaf) were considered infected. We defined plant height as the highest point of the plant measured from the soil and recorded it at the end of the growing season. For these three variables, powdery mildew infection, phenological stage and plant height, we used the maximum values of all tillers per pot or of the five marked plants per plot in glasshouse or field experiment, respectively, for analysis. After ripening, all plants were cut at ground level and separated into vegetative and reproductive parts (spikes). These were then dried at 80 and 25 C°, respectively, and weighed. We then threshed the reproductive parts, counted and removed the seeds infected by ergot (only in field trial) and obtained the total seed mass which is equivalent to the seed yield. The seed number was calculated from the seed yield divided by the average seed mass. The latter was determined on a sample of seeds, one spike in the glasshouse or 1,000 seeds from all spikes in each 1 × 1.08 m plot in the field. The vegetative mass, seed number and seed yield were total measurements of all plants growing in a pot or a plot. Ergot infection rate was calculated as percentage of seed number.

Data analysis

In a factorial design, we grew the eight wheat lines under different fertilizer treatments (three levels in the glasshouse and two in the field). There were five blocks in the glasshouse and four in the field. We analyzed the data of both experiments separately and in combination by analysis of variance (ANOVA). The critical significance level was 0.05 in all analyses. All quantitative pot data from the glasshouse were multiplied by 82.64 to equal an area of 1 m^2. Quantitative field data were divided by 1.08 for the same reason. Regression analysis showed that two variables were slightly affected by the act of vandalism (seed yield: $R^2 = 0.167$ and seed number: $R^2 = 0.094$; n = 64). We removed this effect by multiplying the data of the damaged plots with the negative slope from the regression analysis multiplied by the degree of damage (for 10% damaged area: seed yield: –1.003 g; seed number: –20.8). We used the statistical software GenStat (VSN International Ldt.) to fit multiple regression models and

summarize the results in ANOVA tables for all variables except powdery mildew infection (see Tables S1–S3). Residual plots were examined to identify outliers and to check if the assumptions of normality and homoscedasticity were fulfilled. The vegetative mass of one unusually heavy plant was identified as an outlier and excluded from the analysis. Phenological stage was transformed to the fourth power (y^4); vegetative mass, seed yield and seed number were square-root transformed; and ergot infection rate was cube-root transformed. The binary mildew infection data were analyzed using multiple logistic regression with analysis of deviance (McCullagh and Nelder 1989).

Results

Glasshouse experiment

One half of the replicates in the glasshouse experiment were sprayed with fungicide to simulate environments with and without powdery mildew. While the control lines benefited from the fungicide treatment, the GM lines reacted negatively ($P<0.001$ for GM/control × fungicide interaction). The yield of the GM lines dropped lower than the yield of the sprayed control lines (Figure 1). This indicates that the cost of resistance might be high if the pathogen is absent. Furthermore, sprayed plants showed an acute stress reaction in form of chlorotic leaves. We decided therefore to exclude the sprayed portion of the experiment from further analysis.

The *Pm3b* transgene had the desired phenotypic effect and increased resistance to powdery mildew in the glasshouse experiment (Figure 1; $P<0.001$ for difference GM/control plants, see Table S1). The yield of the GM lines doubled (from 1.60 to 3.23 tonnes per ha^{-1}) compared to the susceptible control lines. GM plants had also more seeds and higher vegetative biomass than control plants in the glasshouse (Figure 2; both $P<0.001$; see Table S2). Phenological development and plant height were not affected by the transgene, indicating that these traits may be genetically more constrained than the other traits.

The four offspring pairs differed significantly from one another in the five fitness-related traits (phenological stage: $P<0.001$, plant height: $P<0.001$, vegetative mass: $P=0.006$, seed number: $P=0.004$, seed yield: $P=0.014$ for main effect of offspring pair). Alternatively, we tested if there was a significant difference between the four control lines. They differed indeed in all traits except the mildew resistance (phenological stage: $P<0.001$, plant height: $P<0.001$, vegetative mass: $P<0.001$, seed

number: P<0.001, seed yield: P<0.001 for the contrast among offspring lines within control). These differences may be caused by the callus culturing of GM and control lines or effects of the transformation itself. Heritable effects acquired in cell culture can have a genetic basis and plants with such effects are sometimes used in plant breeding (Larkin and Scowcroft 1981, Jones 2005).

Depending on the offspring pair, the inserted transgene had significantly different effects on three of the measured traits (Figure 2B; vegetative mass: P=0.012, seed number: P<0.001, seed yield: P<0.001 for GM/control × offspring pair interaction). This suggests that unintended phenotypic effects of the transgene depended on the location where it had been inserted into the genome. In absolute numbers, line *Pm3b*#4 had the highest yield (4.19 tonnes per ha^{-1}) of the four tested GM lines and proved to be highly resistant to powdery mildew (only 20% of plants infected).

Fertilizer application in the glasshouse had positive effects on all traits except phenological stage (Figure 2A). Fertilization also increased mildew infection (P=0.016) which might be due to the increased growth rate of the host plant (Last 1953). Increased nutrient content of the plant material could have boosted the spread of mildew directly (Bainbridge 1974). Differences between GM and control plants generally increased with nutrient level (vegetative mass: P=0.035, seed number: P<0.001, seed yield: P<0.001 for fertilizer × GM/control interaction). We currently have no explanation for this result which demonstrates the importance of testing effects of transgenes across a range of environments.

Field experiment

We measured the same traits in the field experiment as in the glasshouse experiment. In addition we recorded infection by ergot fungus, which occurred naturally in the field but not in the glasshouse. Again, we compared first the four GM lines (*Pm3b*#1–4) with the control lines (Sb#1–4), then the offspring pairs among each other and finally tested the interaction between these two main effects. GM plants with the *Pm3b* transgene showed increased resistance to powdery mildew (Figure 3A and B; P<0.001; see Table S1). In contrast to the glasshouse findings, GM plants had significantly fewer seeds and lower seed yield than control plants (Figure 3A; both P<0.001; see Table S3). Phenological stage, plant height and vegetative mass were not affected by the

transgene. In the field, GM plants showed increased infection by ergot fungus compared with control plants (Figure 4; P<0.001).

The four offspring pairs differed in seed number and their level of ergot infection (seed number: P=0.004, ergot infection: P<0.001 for main effect of offspring pair). Effects of the inserted transgene differed among the four offspring pairs for the dependent variables powdery mildew resistance, ergot infection, seed number and seed yield as reflected in significant GM/control × offspring pair interactions (Figure 3B; powdery mildew infection: P=0.022; ergot infection: P<0.001; seed number: P<0.001, seed yield: P<0.001). That is, in the field, yields of the GM lines *Pm3b*#2 and #4 were reduced by 56% and 48%, respectively, when compared with the corresponding control lines within offspring pairs. The lines *Pm3b*#2 and #4 were completely resistant to powdery mildew in the field, whereas 12.5% of the *Pm3b*#1 and #3 plants were infected. The difference in ergot infection between GM and control lines was small in offspring pair 1 (Figure 4), moderate in offspring pair 3, and large in offspring pairs 2 and 4. Seed infection rates of around 1 %, as found in lines 2 and 4, can reduce grain quality.

In the field, fertilization increased plant height (P=0.006), vegetative mass (P=0.003), seed number (P<0.001) and seed yield (P<0.001). The development of the plants (phenological stage) was not affected by fertilizer application. Similar to the glasshouse, mildew infection increased with fertilizer application in the field (P<0.001). However, in contrast to the glasshouse, fertilization did not alter the difference between the GM and control lines in the field.

Comparison between glasshouse and field experiment

To test if the observed differences in transgene effects between glasshouse and field were statistically significant we also analyzed the datasets from the two experiments together, considering the medium and high nutrient levels in the glasshouse as equivalent to the low and high levels in the field, respectively. As expected, glasshouse and field environments differed significantly from each other. Powdery mildew seemed to favour glasshouse conditions which lead to a stronger infection of the plants in the glasshouse than in the field (P<0.001) thus increasing the potential benefits of resistance caused by the transgene in the glasshouse. Glasshouse plants developed more slowly (phenological stage: P<0.001) and invested slightly more into vegetative mass

(P=0.042) but had fewer seeds (P<0.001) and lower seed yields (P<0.001) than field plants.

GM plants had a fitness advantage over control plants in the glasshouse, but a disadvantage in the field (vegetative mass, seed number and seed yield: P<0.001, plant height: P<0.05 for glasshouse/field × GM/control interaction). While the differences between glasshouse and field could not be assigned to a single environmental factor, the different fertilizer treatments (three levels in the glasshouse and two in the field) did represent such a controlled environmental gradient. We found that fertilizer had similar phenotypic effects in glasshouse and field environments.

Discussion

Transgene × environment interactions

This study demonstrates that GM plants can differ in morphological, fitness- and pathogen-related traits from their control plants. We found several significant transgene (GM vs. control) × environment interactions; that is, depending on the environmental conditions the studied transgene against mildew infection had beneficial or detrimental effects on most of the investigated plant traits. GM plants generally benefited from glasshouse conditions with high mildew infection pressure when compared with control plants but showed a stress reaction when powdery mildew was absent due to fungicide spraying. It is possible that the GM plants lacked the energy to cope with the stress caused by this treatment or the chemical itself could have interacted with the transgene or with pathways involved in *Pm3b*-mediated resistance. It is conceivable that the high fungicide dose increased the extent of the stress reaction of GM plants.

Similar to the fungicide treatment in the glasshouse, the natural conditions outdoors seemed to have stressed the GM plants in the field to the extent that their fitness was significantly reduced. Possible causes of environmental stress in the field were drought and neighbor competition. The only deliberately manipulated factor, i.e. fertilizer application, modified the transgene effects only in the glasshouse but not in the field. Apparently the transgene only offered a relative fitness benefit to GM plants growing under conditions of high mildew incidence but low levels of other stresses. These were exactly the conditions met in the glasshouse but not in the field (nor in the glasshouse after fungicide application). Under less beneficial conditions, the GM plants may have paid a physiological cost for the high intrinsic mildew resistance (Bergelson and Purrington 1996).

Differences among GM lines

The four GM lines, which each contained a single copy of the identical transgene in homozygous condition, differed significantly from each other. There are several potential reasons for these differences. It is possible that cell culturing caused somaclonal variation among the four offspring pairs which subsequently might have interacted differentially with the transgene (Jones 2005). Although theoretically possible (Cubas *et al.* 1999) we would not expect that such interactions would be stably inherited over five plant generations as we found it here. It seems unlikely that random somaclonal events would cause similar effects in two of the four independently transformed lines (*Pm3b*#2 and #4). A more plausible explanation for the differential effects of the inserted transgene among the four offspring pairs may be that positional effects caused the line-specific differences. Several processes are known to cause such effects (Filipecki and Malepszy 2006). Firstly, an inserted transgene may disrupt native genes. Because spring wheat is hexaploid, consists of more than 80% repetitive, non-genic DNA sequences and each GM line was created by a single insertion event, it is unlikely that the disruption of coding genes or their regulatory sequences could have caused these differential effects (Slade *et al.* 2005, Dubcovsky and Dvorak 2007). Secondly, the insertion position of a transgene into the genome may have affected its expression level. Studies have shown that transgene expression rates and activity patterns of independently transformed wheat lines with constitutive ubiquitin promoters can vary (Stoger *et al.* 1999). Depending on the insertion site, flanking DNA regions may partially silence the inserted promoter. Head-to-tail arrangements of the transgenes, in our case of the *Pm3b* and the selectable marker gene, could also have a negative influence on the promoter activity (Rooke *et al.* 2000). It is also possible that in some lines the transgene was inserted into a region of the genome with low transcription activity (Stam *et al.* 1998).

The semi-quantitative expression analysis (Figure S1) indicated that the expression of the *Pm3b* transgene did differ between the four GM lines. Thus, although we lack confirmation by quantitative expression data, it appears that the two GM lines *Pm3b*#2 and #4, where the transgene showed the strongest phenotypic effects, also had the strongest transgene expression. Obviously, this hypothesis should be tested with a much larger number of lines differing in expression levels. However, such a study currently would be beyond our capacities to obtain funding and permissions for field trials. If the hypothesis could be confirmed, there would still be the question whether

the overexpression of the transgene led to an overabundance of its protein product and the subsequent phenotypic effects or if other mechanisms would be involved.

Besides the quantitative reduction of fitness, we observed that some spikes of the two lines *Pm3b*#2 and #4 also differed in their morphology during flowering time and that the same two lines were also more heavily infected by ergot fungus than the other two GM lines and the four control lines. The altered spike morphology may have increased the likelihood of ergot spores entering the florets (Waines and Hegde 2003). However, no indications of altered spike morphology were observed in the glasshouse.

Implications for molecular plant breeding

Although transgenic plant lines with unintended phenotypes commonly arise during molecular plant breeding (Snow *et al.* 2005, Filipecki and Malepszy 2006) they can usually be detected earlier and more easily and are thus not further investigated (Cellini *et al.* 2004) and published. The development of commercial GM plants is based on long selection processes that start in the glasshouse and end in the field. Enormous numbers of seedlings are already discarded before they are exposed to realistic field settings. Our results may have implications for molecular plant breeding: some of the best GM lines in the glasshouse may still show aberrant performance in the field and some not so promising GM lines in the glasshouse may actually be the best for the field. They would likely be lost at early stages of a selection process only targeted at maximum performance under a particular environment. Based on our glasshouse findings, line *Pm3b*#1would have suffered this fate yet was the best in the field. One lesson from our study and from genotype × environment studies in general (Schlichting 1986, Sultan 1987, Schmid 1992, Joshi *et al.* 2001) is that lines which perform particularly well in a specific environment may pay a cost of specialization and perform poorly in other environments.

Conclusions

Our study demonstrates that inserting a single transgene into the hexaploid wheat genome, along with the desired target effect such as mildew resistance in the present case, can significantly affect other phenotypic traits and thus, as in our case, change the ecological behaviour of the species (hypothesis (i) in Introduction). Such unintended effects of single genes to our knowledge are always smaller in experiments using naturally occurring genetic variation and wild plants (Kingsolver *et al.* 2001, Tian *et al.*

2003). Even when we included crop plants, we could not find any publications where single genes reduced quantitative fitness traits in a plant as strongly as in the present case, yet only in the field and not in the glasshouse (Brown 2002). Commercial glyphosate-resistant soybean cultivars were found to suffer from a 5% yield depression that might be caused by the transgene or its insertion process (Elmore *et al.* 2001). One study tested wheat varieties with introduced resistance genes against leaf and stripe rust and reported a 12% reduction of yield (Griffey and Allan 1986), which was considered to be a very large effect (Ortelli *et al.* 1996). Compared with these, the yield reductions of 48 and 56% observed in our two GM lines of wheat expressing the *Pm3b* transgene are much larger (Figure 3B).

We found that the level of mildew resistance as well as the magnitude of other phenotypic effects varied significantly between different GM lines (hypothesis (ii) in Introduction). We hypothesize that this variation in phenotypic effects may be due to different expression levels of the *Pm3b* transgene which in turn might have been caused by different insertion positions of the transgene in the genome. Some plant breeders suggest not selecting for plant lines with complete pathogen resistance because costs of such a resistance often outweigh benefits (Brown 2002). In our case this would speak for selecting GM lines with relatively low expression levels yet still increased mildew resistance, i.e. line *Pm3b*#1 (Masci *et al.* 2003). However, to test the hypothetical correlation between expression level and phenotypic effects would require specific experiments with a larger number of GM lines as used here. With regard to risk assessment our findings are in agreement with the view that each GM line should be tested in a case-by-case approach (Andow and Zwahlen 2006).

Finally, our results show that even if desired phenotypic effects of a transgene are found across a range of environments in a glasshouse experiment, some of these effects can be reversed if GM lines are exposed to natural environmental variation in the field (hypothesis (iii) in Introduction). Although it is likely that commercial plant breeders know of the presence of transgene × environment interactions, it seems that such observations so far have not found their way into the scientific literature. Breeding trials to select lines for further investigation do not need full replication and randomization, yet for an assessment of the ecological behaviour of such lines, replicated and randomized ecological experiments would be required. Our study may serve as an example of potential results that can be obtained in such experiments. We

believe that such experiments can help us to gain a deeper understanding of single-gene effects in plant ecology and evolution.

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Figures

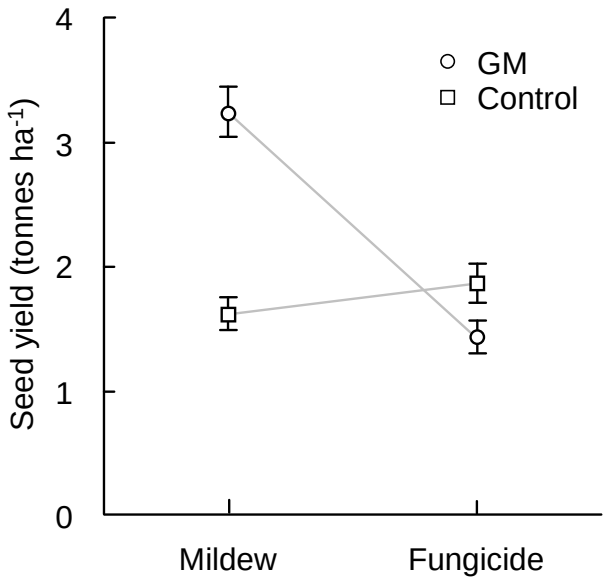

Figure 1. Effects of mildew infection and fungicide spraying on yields of GM wheat lines. Example of significant transgene × environment (presence/absence of powdery mildew) interaction in GM spring wheat in a glasshouse experiment. GM plants (circles = *Pm3b*#1 to #4) have higher yield than control plants (squares = Sb#1–4) in the presence but lower yield in the absence of mildew (fungicide spraying); light grey lines were drawn to make interactions between transgene and environments visible; error bars represent ± 1 standard error (back-transformed from square root scale).

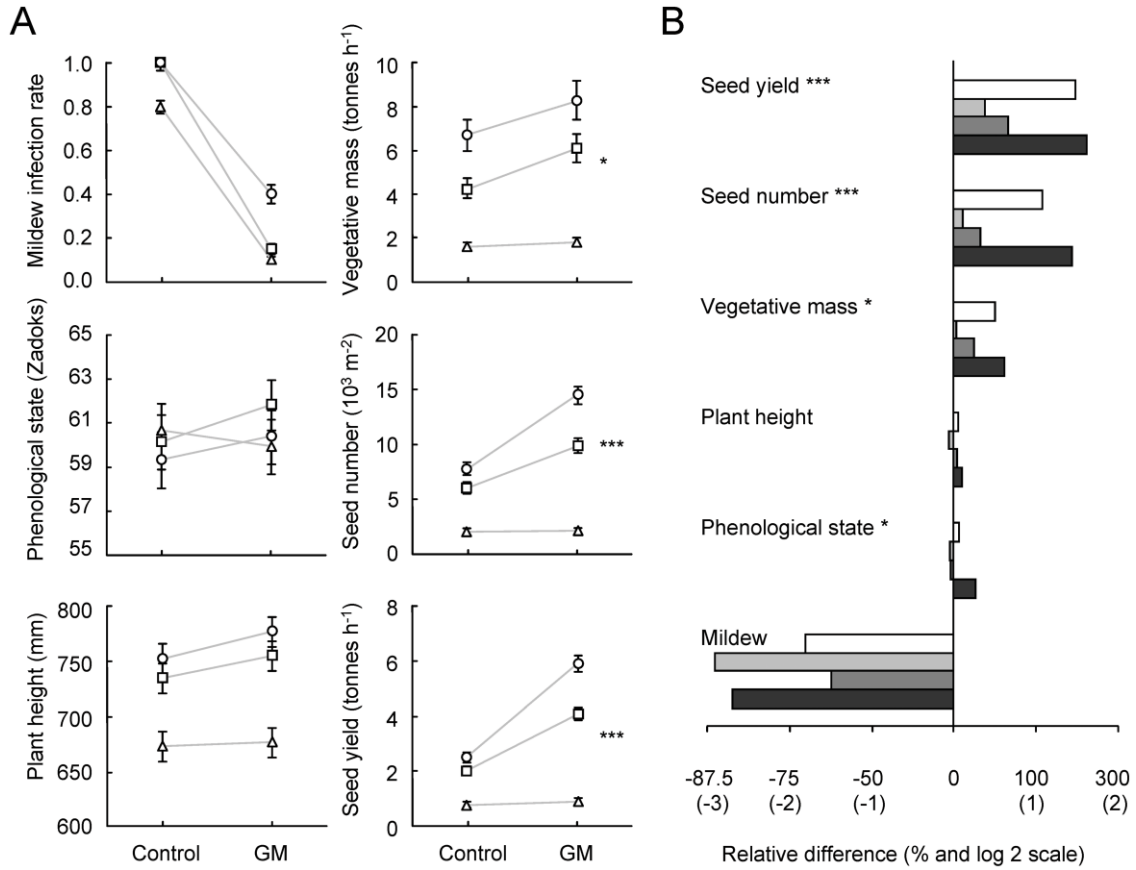

Figure 2. Effects of the transgene in the glasshouse on mildew infection and plant performance traits. The mildew infection equals the proportion of pots with strong powdery mildew infection up to flag leaves. Phenological stage, plant height, vegetative mass, seed number and seed yield were measured to assess the plant performance. A: mean of four lines (Control = Sb#1–4; GM = *Pm3b*#1–4) at different soil nutrient levels (circles = high fertilizer, squares = medium fertilizer, triangles = no additional fertilizer); significant transgene × fertilizer environment interactions indicated by asterisks (vegetative mass: P=0.035, seed number: P<0.001, seed yield: P<0.001); light grey lines were drawn to make these interactions visible; error bars represent ± 1 standard error (back-transformed, see methods) and are sometimes hidden behind the symbols. B: proportional difference between GM and control plants for each of the four offspring lines but averaged across nutrient levels (white bars = offspring pair 1 (*Pm3b*#1 *vs.* Sb#1), light grey = offspring pair 2, dark grey = offspring pair 3, black bars = offspring pair 4); x-axis log-scale with original values (100 * GM/control); bars extending to the right from the vertical zero line indicate higher values in GM than in control plants; significant GM/control × offspring pair interactions indicated by asterisks (* P<0.05; ***P<0.001).

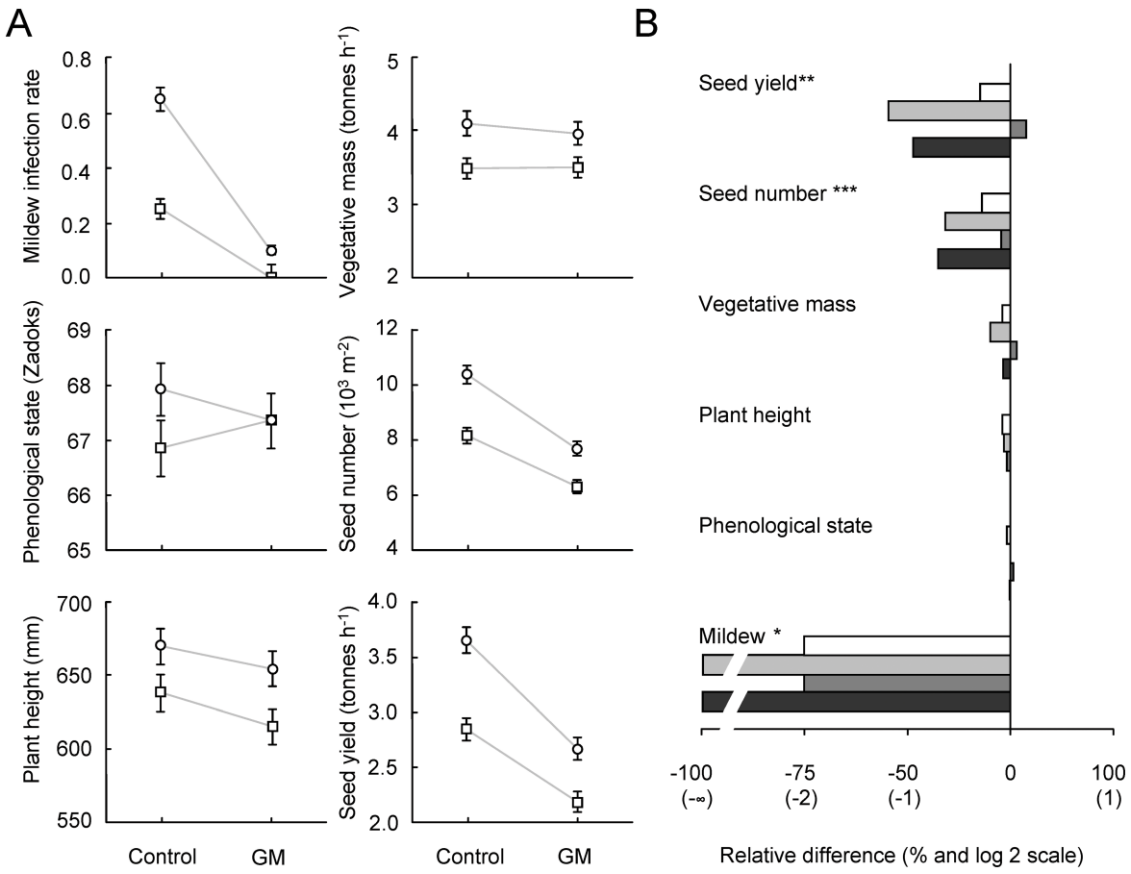

Figure 3. Effects of the transgene in the field on mildew infection and plant performance traits. The mildew infection equals the proportion of pots with strong powdery mildew infection up to flag leaves. Phenological stage, plant height, vegetative mass, seed number and seed yield were measured to assess the plant performance. A: mean of four lines at different soil nutrient levels (circles = additional fertilizer, squares = no fertilizer); transgene × fertilizer environment interactions were never significant; light grey lines were drawn to make this visible; error bars represent ± 1 standard error (back-transformed, see methods). B: proportional difference between GM and control plants for each of the four offspring lines but averaged across nutrient levels (white bars = offspring pair 1 (*Pm3b*#1 *vs.* Sb#1), light grey = offspring pair 2, dark grey = offspring pair 3, black bars = offspring pair 4); x-axis log-scale with original values (100 * GM/control); bars extending to the right from the vertical zero line indicate higher values in GM than in control plants; significant GM/control × offspring pair interactions indicated by asterisks (* P<0.05; ** P<0.01; ***P<0.001).

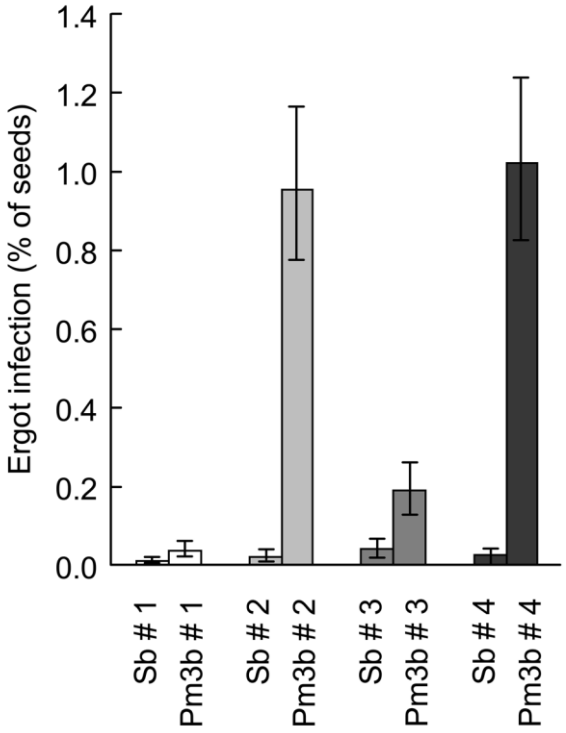

Figure 4. Percentage of ergot infected seeds in GM and control plants in the field. White bars = offspring pair 1, light grey = offspring pair 2, dark grey = offspring pair 3, black bars = offspring pair 4. Within each pair, the bar to the left shows control line and the bar to the right shows the corresponding GM line. Error bars represent ± 1 standard error (back-transformed from cube root scale.

Supplemental Material to Chapter 3

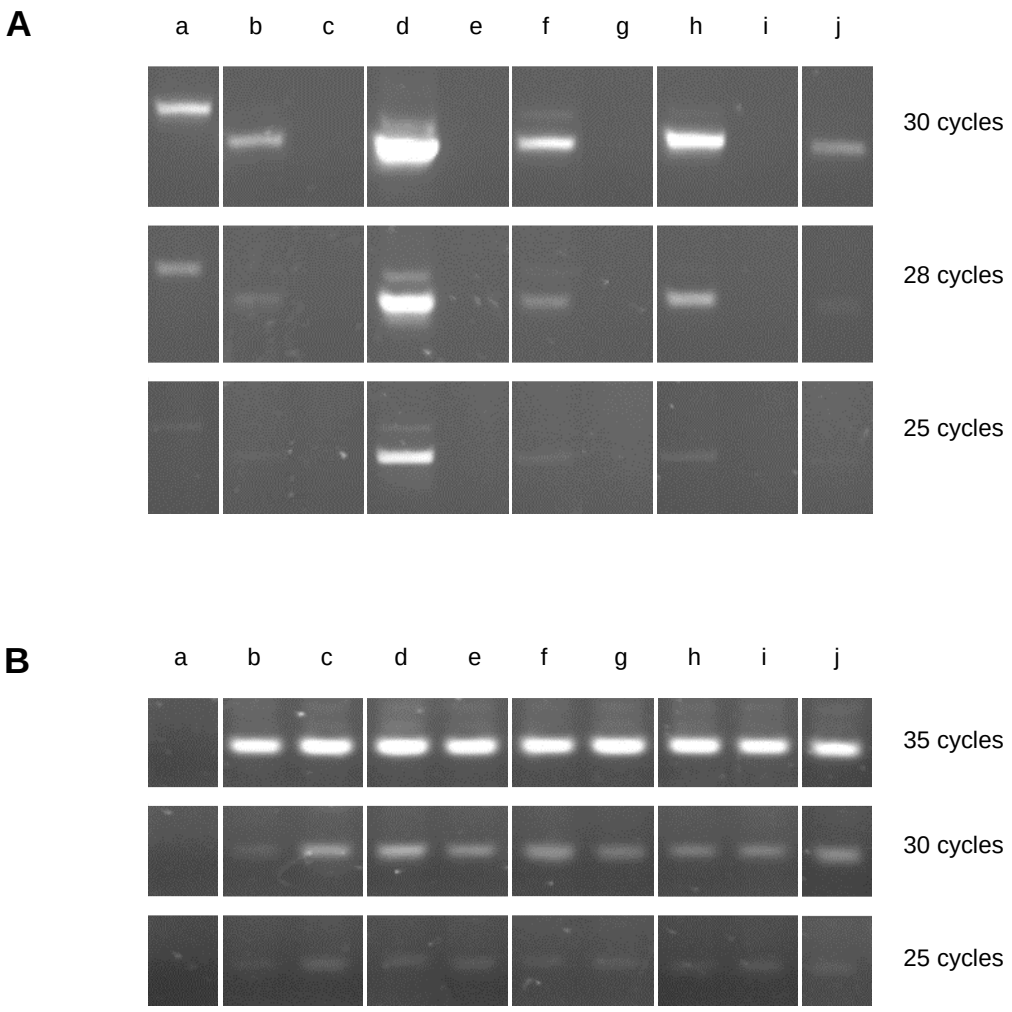

Figure S1. Semiquantitative expression analysis of *Pm3b* and *Mlo* in GM wheat lines. These gel photographs show semi-quantitative PCR expression analyses. A: Analysis of *Pm3b* expression in the *T. aestivum* lines *Pm3b*#1–4 (b, d, f, h) and the corresponding control lines Sb#1–4 (c, e, g, i). As positive controls, genomic DNA (a) and cDNA (j) of the variety Chul carrying one endogenous copy of *Pm3b* were used. The number of PCR cycles is indicated on the right. The photographs of the gel were cropped and rearranged graphically. B: As control for equal amount and quality of template cDNA, the expression levels of the *Mlo* gene were determined. Negative control water (a), *Pm3b*#1–4 (b, d, f, h), corresponding control lines Sb#1–4 (c, e, g, i), variety Chul (j).

Table S1. This ANOVA table shows the effect of the Fertilizer, GM / control, Offspring pair treatments and their interactions on the phenological stage, plant height, vegetative mass, seed number and seed yield in the glasshouse experiment.

		Phenological stage		Plant height		Vegetative mass		Seed number		Seed yield	
Source of variation	df	% SS	F pr.	% SS	F pr.	% SS	F pr.	% SS	F pr.	% SS	F pr.
Block	4	7.1	0.018	0.4	0.933	0.2	0.534	0.7	0.374	0.6	0.379
Fertilizer	2	0.5	0.651	26.3	<.001	86.1	<.001	61.8	<.001	55.6	<.001
GM / control	1	0.3	0.500	1.2	0.142	2.8	<.001	8.2	<.001	16.3	<.001
Offspring pair	3	26.4	<.001	15.8	<.001	1.0	0.006	2.5	0.004	1.6	0.014
GM / control × Offspring pair	3	6.0	0.017	3.4	0.099	0.9	0.012	4.7	<.001	3.9	<.001
Fertilizer × GM / control	2	0.6	0.586	0.4	0.720	0.5	0.035	4.1	<.001	6.8	<.001
Fertilizer × Offspring pair	6	3.5	0.410	0.6	0.979	0.5	0.394	0.9	0.536	0.5	0.717
Fertilizer × GM / control × Offspring pair	6	3.8	0.350	3.0	0.479	0.6	0.286	1.2	0.319	1.3	0.197
Residual	92	51.8		49.0		7.3		15.8		13.3	
Total	119	100.0		100.0		100.0		100.0		100.0	
		x^4 transformed				Sqrt transformed		Sqrt transformed 1 plant without seeds excluded		Sqrt transformed 1 plant without seeds excluded	

Table S2. This ANOVA table shows the effect of the Fertilizer, GM / control, Offspring pair treatments and their interactions on the phenological stage, plant height, vegetative mass, seed number, seed yield and ergot infected seeds in the field experiment.

Source of variation		Phenological stage		Plant height		Vegetative mass		Seed number		Seed yield		Ergot Infection	
	df	% SS	F pr.	% SS	F pr.	% SS	F pr.	% SS	F pr.	% SS	F pr.	% SS	F pr.
Block	3	69.1	<.001	3.0	0.583	3.7	0.511	3.7	0.073	4.9	0.035	2.1	0.229
Fertilizer	1	0.6	0.288	12.7	0.006	15.2	0.003	20.0	<.001	19.4	<.001	0.1	0.719
GM / control	1	0.0	0.935	3.7	0.126	0.1	0.761	32.3	<.001	31.7	<.001	40.3	<.001
Offspring pair	3	3.7	0.083	1.8	0.759	3.5	0.538	7.5	0.004	3.5	0.097	20.0	<.001
GM / control × Offspring pair	3	1.5	0.418	1.7	0.768	3.1	0.592	11.9	<.001	14.1	<.001	14.8	<.001
Fertilizer × GM / control	1	0.6	0.288	0.1	0.754	0.2	0.702	0.6	0.271	0.6	0.284	0.3	0.415
Fertilizer × Offspring pair	3	0.7	0.720	6.6	0.239	1.6	0.806	0.3	0.908	0.4	0.852	0.8	0.625
Fertilizer × GM / control × Offspring pair	3	0.7	0.720	2.4	0.665	0.7	0.936	1.5	0.397	1.7	0.364	0.3	0.884
Residual	45	23.2		68.0		71.8		22.2		23.6		21.3	
Total	63	100.0		100.0		100.0		100.0		100.0		100.0	
		x^4 transformed				Square root transformed		Square root transformed		Square root transformed		Cube root transformed	

Table S3. These ANOVA table shows the effect of the Fertilizer, GM / control, Offspring pair treatments and their interactions (3 way interaction omitted) on the mildew infection rate in the glasshouse and field experiment.

Source of variation	Glasshouse			Field		
	df	% SS	F pr.	df	% SS	F pr.
Block	4	0.5	0.854	3	2.1	0.413
Fertilizer	2	3.2	0.016	1	9.8	<.001
GM / control	1	48.4	<.001	1	32.0	<.001
Offspring pair	3	1.8	0.186	3	6.1	0.051
GM / control × Offspring pair	3	2.0	0.15	3	7.8	0.022
Fertilizer × GM / control	2	2.3	0.052	1	0.3	0.538
Fertilizer × Offspring pair	6	5.4	0.032	3	6.6	0.039
Residual	98	36.4		48	35.2	
Total	119	100.0		63	100.0	

CHAPTER 4

Mixtures of Genetically Modified Wheat Lines Outperform Monocultures

Simon L. Zeller, Olena Kalinina, Dan F. B. Flynn, Bernhard Schmid. 2012. Ecological Applications 22:1817-1826

Mixture plot of Bobwhite GM wheat lines

Abstract

Biodiversity research shows that diverse plant communities are more stable and productive than monocultures. Similarly, populations in which genotypes with different pathogen resistance are mixed may have lower pathogen levels and thus higher productivity than genetically uniform populations. We used genetically modified (GM) wheat as a model system to test this prediction, because it allowed us to use genotypes that differed only in the trait pathogen resistance but were otherwise identical. We grew three such genotypes or lines in monocultures or two-line mixtures. Phenotypic measurements were taken at the level of individual plants and of entire plots (population level). We found that resistance to mildew increased with both GM richness (0, 1 or 2 *Pm3* transgenes with different resistance specificities per plot) and GM concentration (0, 50 or 100% of all plants in a plot with a *Pm3* transgene). Plots with two transgenes had 34.6% less mildew infection and as a consequence 7.3% higher seed yield than plots with one transgene. We conclude that combining genetic modification with mixed cropping techniques could be a promising approach to increase sustainability and productivity in agricultural systems, as the fitness cost of stacking transgenes within individuals may thus be avoided.

Introduction

Since the mid-20th century, the Green Revolution allowed agricultural yields to increase continuously, for example, in bread wheat in Europe from about 1.5 t in 1950 to 7 t of grain per ha in 1996, but since then wheat yields have stagnated (Brisson *et al.* 2010). Fertilizer, pesticides and new crop varieties contributed to the dramatic increases in yields (Conway 1997). However, the impact of this development on the environment has also been considerable and unfortunately often negative (Tilman *et al.* 2001). Organic farming, on the other hand, has allowed a reduction of the input of agrochemicals but only at the cost of reduced yields (Maeder *et al.* 2002).

Genetic engineering may hold solutions to this problem. For example, crop plants with introduced resistance traits may help to reduce pesticide use while maintaining or even increasing yields (Borlaug 2000). Some of these genetically modified (GM) crops have been so successful that they are currently planted on large areas (James 2009). This leads to a high selection pressure on the pests to overcome the resistance by evolution of new genotypes (Tabashnik *et al.* 2009, Powles 2010), which in turn may reduce the advantages of GM crops. Efforts are being made to slow down the evolution of such new pest genotypes. Besides refuge strategies, the combination of several GM traits within a single plant, also known as pyramiding or stacking, has been promoted (Bravo and Soberon 2008). However, the sustainability of this approach might be compromised, as "super-pests" may evolve that overcome such multiple resistance, particularly if single-transgene and multiple-transgene crops are planted in close proximity (Zhao *et al.* 2005). Another problem, which to date has rarely been addressed, are potentially increased defense costs that multiple resistances impose on an individual plant (Kalinina *et al.* 2011).

Here we suggest that one solution to these problems could be using mixtures of lines with different but complementary resistance traits, i.e., stacking genes at the population rather than the individual plant level. In addition to increasing resistance at the population level, such a strategy should allow the different pathogen strains to survive in low numbers on some plants, thus reducing the selection pressure on the pathogen to overcome plant resistance.

Ecological theory and results of recent biodiversity experiments suggest this line of argumentation. In grassland biodiversity experiments, productivity generally increases with diversity (Tilman *et al.* 1996, Hector *et al.* 1999, Roscher *et al.* 2005).

Such increased productivity of total biomass in grasslands with plant diversity has some analogs with increased yield in agricultural systems. One of the reasons for increased yield with plant diversity in agricultural systems is reduced pathogen susceptibility (Zhu *et al.* 2000). For example, wheat lines susceptible to mildew have lower levels of infection if they are surrounded by resistant lines (Kalinina *et al.* 2011). Particular pathogens are less likely to become dominant in a diverse system when their particular hosts all occur at low abundance (Keesing *et al.* 2006). Only generalist pathogens would be able to thrive in diverse systems of hosts, and such generalists may be less efficient in overcoming the defense of a particular host due to trade-offs among the different adaptations needed to overcome the defenses of a diverse set of hosts (Woolhouse *et al.* 2001).

While ecologists are currently investigating the mechanisms by which species-rich plant communities have lower pathogen abundance and higher yields (Maron *et al.* 2011), agronomists came across similar phenomena some time ago, albeit at the between-variety, within-species level. Mixtures of several varieties of the same crop species can have higher yields than monocultures of single varieties (Browning and Frey 1969, Wolfe 1985). However, diversity strategies have rarely been used so far for technical reasons, such as uniformity requirements for varieties and seed material and harvesting efficiency (Smithson and Lenne 1996). In part these technical difficulties may be overcome with better harvesting technology. Another and probably easier solution would be to produce plants by genetic engineering that only differ in the resistance traits of interest. Fields with mixed lines would then still have uniform phenology and harvest traits and could be easily harvested.

We experimentally compared wheat *Triticum aestivum* L. plots consisting of single lines with mixed plots. The lines differed only in their resistance to powdery mildew *Blumeria graminis* f.sp. *tritici* (DC.) Speer, which was possible due to the introduction of a single gene using gene technology. One non-transgenic control line and two transgenic (GM) lines of spring wheat variety Bobwhite were used in the experiment. Mildew infection, plant production and seed yield were assessed at the level of the individual plants and the plot to test their response to increasing GM richness (0, 1, or 2 GM lines) and GM concentration (0, 50, 100% of individuals from GM lines) of the plots. Our hypotheses are as follows:

- H1: If plot-level transgene diversity reduces powdery mildew infection more efficiently than transgene monocultures, both higher GM concentration and especially higher GM richness will reduce powdery mildew infection.
- H2: Such reductions in powdery mildew will increase seed yield at the plot level.
- H3: If the underlying mechanism for the transgene diversity effect is mediated by the density of plants, then the effect of diversity will be significant for plant performance at the plot level rather than at the individual level, since results from individual plants will not be effective predictors of plot-level responses.
- H4: In contrast, if the mechanism is for individual plants to have reduced risk of infection as transgene diversity increases, then the effect of diversity will be significant at the level of individual plant performance.

Materials and Methods

Genetically modified wheat

We used two transgenic wheat lines, derived from different transformation events of Bobwhite SH 98 26 and carrying transgenes *Pm3a* or *Pm3b*, and the control line Bobwhite SH 98 26 (Peter *et al.* 2010, von Burg *et al.* 2010, Zeller *et al.* 2010, Brunner *et al.* 2011). These transgenes confer different race-specific resistances to powdery mildew and were cloned from hexaploid wheat (Yahiaoui *et al.* 2004, Srichumpa *et al.* 2005). *Pm3a* and *Pm3b* were originally isolated from the wheat varieties Asosan and Chul, respectively. Two lines carrying one of the two genes each were generated by biolistic transformation of spring wheat variety Bobwhite SH 98 26 (Pellegrineschi *et al.* 2002). The generation and selection of line *Pm3b*#1 has been described in detail before (Zeller *et al.* 2010, Brunner *et al.* 2011). Similar protocols were used to generate the line *Pm3a*#1 (S. Brunner, personal communication). For simplicity, these two lines will be named *Pm3a* and *Pm3b*, respectively, throughout this paper. The *Pm3a* and *Pm3b* genes were cloned under the control of the *Zea mays* L. (maize) ubiquitin promoter (Christensen and Quail 1996) and transformants were selected on mannose-containing media using the phosphomannose isomerase (PMI)-coding gene as selectable marker (Reed *et al.* 2001). Southern hybridization analysis (Southern 2006) showed that *Pm3a* carried two and *Pm3b* one copy of the corresponding *Pm3* transgene.

The seeds used in this study were obtained from GM lines that had passed through four (*Pm3*a) or five (*Pm3b*) generations of sexual reproduction.

The expression level of the *Pm3a* and *Pm3b* transgenes in the two GM lines was assessed by qRT-PCR using RNA isolated from leaves collected during the field trial in 2009. *Pm3a* was 6–45 times and *Pm3b* 11–130 times more highly expressed in the GM lines than in wheat line Chul which harbors the *Pm3b* gene naturally (Brunner *et al.* 2011 and S. Brunner personal communication).

Field experiment

The field experiment took place at an agricultural research station in Zurich-Reckenholz, Switzerland, from March–July 2009. Four replicate blocks, each with six 3 × 1.08 m plots, were sown with *Pm3a, Pm3b* and Bobwhite SH 98 26 monocultures and the three 1:1 mixtures *Pm3a*/Bobwhite, *Pm3b*/Bobwhite and *Pm3a*/*Pm3b*. In each plot, 400 seeds were sown in six rows with a distance of 17.8 cm between rows using an Oyjord plot drill system (Wintersteiger AG, Ried, Austria). The experimental plots were alternated with triticale plots in a chessboard-like design to eliminate possible neighbor effects. To allow uniform infection by powdery mildew, single rows of the susceptible winter wheat variety Kanzler were planted on both sides of each plot. Powdery mildew infection occurred naturally and evenly throughout the experiment.

All seeds were treated with the fungicide Jockey (167g l^{-1} Fluquinconazole, 34 g l^{-1} Prochloraz; Omya Agro AG, Safenwil, Switzerland) before sowing. The amount of mineralized nitrogen, determined at the end of February in the top 100 cm of the soil, was 35.1 and 47.6 kg N ha^{-1} in blocks 1/2 and 3/4, respectively. Nitrogen fertilizer was applied the day before sowing (40 kg N ha^{-1} in blocks 1/2, 30 kg N ha^{-1} in blocks 3/4) and again 30 kg N ha^{-1} ("Ammonsalpeter 27.5", Lonza, Visp, Switzerland) at the phenological stage 22–29 (Zadoks *et al.* 1974). The natural field soil provided the plants with sufficient phosphorous, potassium and magnesium (75, 182 and 213 mg kg^{-1}). All plots were sprayed with the herbicide cocktail Concert SX (40% Thifensulfurone, 4% Metusulfurone-methyl; Stähler Suisse AG, Zofingen, Switzerland) and Starane super (120 g l^{-1} Bromoxynil, 120 g l^{-1} Ioxynil, 100 g l^{-1} Fluroxypyr-metilheptil-ester; Omya Agro AG, Safenwil, Switzerland) at the beginning of May. All plots were treated twice with the insecticide Karate Zeon (100 g l^{-1} Lambda-Cyhalothrin; Syngenta Agro AG, Dielsdorf, Switzerland) against the wheat stem fly (*Chlorops pumilionis* Bjerk.) at the beginning of May and 2 weeks later. Due to weed

infestation the whole trial was sprayed with Puma Extra (69 g l^{-1} Fenoxaprop-P-ethyl, 75 g l^{-1} Mefenpyr-Diethyl; Omya Agro AG, Safenwil, Switzerland).

In each plot, 10 individual plants were marked shortly after germination. These individuals were distributed evenly over the 3 m plot length and randomly among the four inner rows. This allowed us to obtain a representative sample of the entire plot while excluding edge effects.

Response variables

To address the hypotheses that plant response at the plot level (H3) is the most indicative of infection rates in response to transgene diversity or that the response of individual plants (H4) can be an effective proxy of such plot-level responses, we measured six phenotypic traits on individual plants and five traits on entire plots. Individual plants were assessed for the degree of powdery mildew infection (Eyal *et al.* 1987) 44, 59 and 78 days after germination. Based on these time points, the Area Under Disease Progress Curve (AUDPC) was calculated (Jeger and Viljanen-Rollinson 2001). Furthermore, phenological stage (Zadoks *et al.* 1974) and height were assessed 59 and 78 days, respectively, after germination for each plant. The Zadoks scale allows classifying individual cereal plants or entire plots into development stages reaching from 1 (start of germination) to 99 (ripening complete). At the end of the growing season, height was recorded again and then all individual plants were cut at ground level and separated into vegetative and reproductive parts (spikes). Vegetative and reproductive parts were dried at 80 and 25 C°, respectively, and weighed. The reproductive parts were threshed to obtain seeds and determine total seed mass per plant, here referred to as individual seed production. Finally, the seed mass of the individual plants was divided by the number of seeds and multiplied by one thousand to calculate the thousand seed weight (TSW).

Two non-destructive measurements were conducted at the plot level. Leaf Area Index (LAI) was measured on the western side of each plot 25 and 35 days after germination (LAI 2000 Plant Canopy Analyser, LI-COR Biosciences; Lincoln, USA). It consisted of two measurements close to an inner row and one between the rows as well as a control measurement above the canopy. To assess differences in flowering time, the percent of plants with flowering spikes in each plot was determined 64 days after germination. At this time, all plots had flowering spikes. A subplot of 50 × 72.2

cm was harvested in the same place were the LAI was measured in each plot. These subplots were placed 50 cm from the western edge of the plot and excluded the two outer rows. The harvested material was separated into vegetative and reproductive parts to determine biomass, seed yield and thousand seed weight at plot level. The latter was determined on a sample of 1000 seeds.

Data analysis

We analyzed the data of individual plants and plots separately by mixed-model analysis of variance using the REML (Restricted Maximum Likelihood) method. We used the statistical software GenStat (VSN International Ldt.). The critical significance level was 0.05 in all analyses. However, we also present and discuss some results which were marginally significant at the 0.1 level (Peto *et al.* 1976, Toft and Shea 1983). The results of the mixed-model analyses are summarized in tables for all variables (see Appendix A and B). Residual plots were examined to identify outliers and to check if the assumptions of normality and homoscedasticity were fulfilled. For the six diversity treatments (three monocultures and three mixtures), two linear but non-orthogonal contrasts were made to test for effects of increasing GM richness (0 for monoculture Bobwhite control, 1 for each of the two GM monocultures and the mixtures of each GM with Bobwhite control, 2 for the mixture of the two GM) or increasing GM concentration (0% for monoculture Bobwhite, 50% for each of the 2 mixtures of one GM and Bobwhite control, 100% for the two GM monocultures and the mixture of the two GM). Since these two contrasts were partly confounded with each other, their fitting sequence was swapped in two alternative statistical models. For GM richness, which was the focus of our study, the different sequences can be interpreted as follows: when GM richness is fitted first, confounding effects of GM concentration are ignored; when GM richness is fitted second, it measures the difference between richness levels corrected for increasing GM concentration. Predicted means and standard errors from the REML-output were used to draw figures.

Since several of the measured traits were correlated with each other, we also performed a multivariate analysis of variance (MANOVA) to test for the overall significance of treatment effects. For the individual plant data the six traits, AUDPC, phenological state, plant height, biomass, seed mass and TSW, were included in the MANOVA. For the plot data the five traits, LAI, flowering time, biomass, seed mass and TSW, were included in the MANOVA.

To directly compare mixtures with monocultures of wheat lines, a deviation or D-value (Loreau 1998) was calculated separately for each plot containing a line mixture in each block. For this calculation, the mean of the two monocultures was first subtracted from the mixture and the resulting value then divided by the mean of the two monocultures. A D-value greater than 0 indicates, for example, that the yield of a mixture is higher than what would be expected from the mean of the monocultures. The opposite would be true for a negative D-value. We calculated D values for powdery mildew infection, plot biomass, seed yield, and TSW.

To investigate mechanisms that might explain the observed treatment effects in one response variable, we tested the other, earlier-measured response variables as covariates. Powdery mildew infection had the best explanatory power for variation in the other traits and thus results of REML models with this covariate are also presented.

Results

Individual-level responses

The multivariate analysis for the individual plant data showed highly significant effects of the diversity treatment ($P<0.001$, Appendix A). These were also reflected in significant GM richness or GM concentration contrasts ($P = 0.002$ for each if fitted first) and significant differences between plots containing either *Pm3a* or *Pm3b* ($P=0.001$). Following the finding of significant effects overall for transgene diversity on multivariate plant responses, each response was then analyzed individually.

Powdery mildew infection as measured by AUDPC at the individual plant level decreased with increasing GM richness and GM concentration of plots (Figure 1A; $P<0.001$; see Table S1 in Supplemental Material). Both contrasts were highly significant if fitted first (GM richness: $P<0.001$; GM concentration: $P<0.001$) or second (GM richness: $P=0.038$; GM concentration: $P=0.031$) in the statistical model. Plots containing two GM lines had 65.1% and plots containing one GM line had 31.7% lower mildew infection than non-transgenic control plots. Plots with 50% GM plants had 31.7% and plots with 100% GM plants had 52.8% lower mildew infection than plots without GM plants. No significant difference between the two GM lines *Pm3a* and *Pm3b* was detected ($P=0.141$). All mixtures were less infected by mildew than expected from the means of the monocultures. D-values were –0.072, –0.144 and –0.345 for the mixtures BW/*Pm3a*, BW/*Pm3b* and *Pm3a/Pm3b*, respectively. This means that plants

in plots with BW/*Pm3a* had 0.3%, plots with BW/*Pm3b* 20.7% and plots with both GM lines had 34.6% less powdery mildew than expected from the corresponding monoculture means.

The phenological development of GM plants measured 59 days after germination was on average not significantly different from that of control plants (Figure 1B and Table S1 in Supplemental Material). However, *Pm3b* developed significantly faster than *Pm3a* (difference = 2.2 points on Zadoks Scale, P<0.001). This means that an introduced transgene can influence the phenological development of a plant.

Individual plants in Bobwhite control plots were significantly shorter than in plots harboring GM plants (Figure 1C; difference = 3.8cm; P=0.014). Plant height increased with GM richness and GM concentration (sum of the two contrasts significant at P=0.013). However, the individual contrasts were only significant if fitted first in the statistical model (GM richness: P=0.013; GM concentration: P=0.013).

Pm3a had significantly more biomass than *Pm3b* (Figure 1D; difference= 0.55 g/plant; P=0.036). There was a trend towards higher biomass with increased GM richness (P=0.099) but GM concentration did not influence the biomass of individual plants. *Pm3a* had a marginally higher individual seed production than *Pm3b* (P=0.055) and GM richness marginally increased individual seed production as well (P=0.092). *Pm3a* had significantly more (data not shown, P=0.003) but lighter seeds than *Pm3b* (Figure 1F, difference = 5.4 g TSW; P=0.003). TSW increased with either GM richness or GM concentration if the corresponding contrast was fitted first in the statistical model (GM richness: P=0.023; GM concentration: P=0.047) but not if it was fitted second.

Plot-level responses

In the multivariate analysis with the plot-level data the diversity treatment effects were also highly significant (P=0.002, Table S2 in Supplemental Material). GM concentration was significant if fitted first or second (P=0.021 and P=0.005). GM richness, however, was only significant if fitted second, i.e. after GM concentration (P=0.020), indicating that after correction for increasing GM concentration, plots with two GM lines differed from plots with only one GM line. Furthermore, plots containing *Pm3a* differed significantly from plots containing *Pm3b* (P<0.001).

The LAI measured at the beginning of the growing season (25 days after germination) decreased with increasing GM concentration (Figure 2A and Table S2 in Supplemental Material; GM concentration: P=0.01 if fitted first and P=0.028 if fitted second). However, this effect disappeared 35 days after germination. On day 64 after germination, plots with high GM concentration had fewer flowering spikes than plots with low GM concentration (Figure 2B; P=0.005). Fitted after GM concentration, GM richness also affected the number of flowering spikes (P=0.012). Furthermore, plots with *Pm3a* had significantly fewer flowering spikes than plots with *Pm3b* (P<0.001). This result is consistent with the individual plant data, where *Pm3a* was shown to develop more slowly than *Pm3b*.

The aboveground biomass in the plots did not differ statistically significantly among the six diversity treatments (Figure 2C). However, a positive D-value of 0.062 indicated that the GM-GM mixture tended to have higher biomass than expected from the mean of the two GM monocultures. Clearer differences were found for seed yield (Figure 2D). Plots with high GM richness had higher yield than plots with low GM richness (P=0.04). In numerical values plots with two GM lines had a 16.7% higher seed yield than control lines whereas plots with only one GM line only had a 5.4% higher seed yield than control lines. A positive D-value of 0.073 indicated that the GM-GM mixture performed 7.3% better than expected from the mean of the two GM monocultures. Since the mixture was also producing a higher seed yield than the better GM monoculture, there was evidence for transgressive overyielding (Schmid *et al.* 2008).

The TSW increased significantly with GM richness (Figure 2E, P=0.006). Seeds from plots with two GM lines were 11.9% heavier than seeds from control plots, whereas seeds from plots with only one GM line were only 5.6% heavier than seeds from control plots. This was also reflected in positive D-values for all mixtures. Similar to the individual plant data, seeds from plots containing *Pm3b* were significantly heavier than seeds from plots containing *Pm3a* (P=0.016).

Analyses with covariate mildew infection

To assess the influence of the mildew infection on other measured traits we repeated the analysis with AUDPC as covariate. On the individual plant level, plant height and TSW were affected significantly (plant height: P=0.001; TSW: P=0.002) by AUDPC. The

inclusion of the covariate fully explained the effects of GM richness and concentration on plant height and TSW. Thus the two contrasts were no longer significant if fitted after the covariate. However, the differences between lines *Pm3a* and *Pm3b* persisted.

At the plot level, biomass, seed yield and TSW were significantly influenced by the covariate. Whereas the covariate did not remove the significance of the remaining effects on plot biomass, it did explain the GM richness and concentration effects on seed yield and TSW at plot level, which both were no longer significant if fitted after the covariate. However, the differences between plots containing line *Pm3a* vs. *Pm3b* remained significant. Overall, these results suggest that the reduced mildew infection found in plots with high GM richness or GM concentration had a positive influence on plant height, seed yield and TWS.

Discussion

Mixing GM lines reduces mildew infection (H1) and increases yield (H2)

This study demonstrates that genetically modified (GM) wheat plants perform differently when grown in mixtures with other GM lines or control lines than when grown in single-line monocultures. The performance of individual plants and of entire plots generally increased with the number of GM lines (GM richness, ranging from 0–1–2) or with the proportion of GM plants (GM concentration, ranging from 0–50–100%) in a plot. Thus, powdery mildew resistance increased with GM concentration, indicating that the transgene worked as expected. Furthermore, mildew resistance also increased with GM richness. This was probably due to the fact that the two GM lines harbored transgenes that were effective against different races of powdery mildew and thus they could complement each other in mixture and provide resistance against a wider spectrum of pathogens than if the same lines were grown in single-line mixtures. This indicates that a diversity of resistance transgenes can have a beneficial effect at the plot level, avoiding the need to stack these genes in each single plant, potentially leading to higher fitness costs (Kalinina *et al.* 2011). If in mixtures a certain proportion of individual plants are resistant against a specific pathogen they can reduce the spread of infection (Browning and Frey 1969, Schmid 1994). Not only mixtures of two GM lines, but also mixtures of a GM line with a control line were less infected with powdery mildew than expected from the means of the two monocultures. In this case as well, the non-resistant plants of the control line may have profited from the protection by neighboring resistant GM plants.

Besides the resistance to powdery mildew, we assessed a number of phenotypic traits correlated with performance. Individual plants grew taller and produced larger seeds in plots with increased GM richness or concentration. However, at the plot level we recorded a lower leaf area index at the beginning of the growing season and a later flowering time in plots with high GM concentration. This could indicate costs of resistance (Bergelson and Purrington 1996). Nevertheless, seed size and seed yield increased with GM richness: one of the two plots with a GM/control line mixture (*Pm3b*/BW) increased its yield by 3.8% compared the mean of single monocultures. Because the seed yield of the mixture of the two GM lines was even higher than that of the better single-GM line monoculture (yield of *Pm3b*/*Pm3a* mixture was 6.5% higher than in *Pm3b*), this can be considered as one of the rare cases of transgressive overyielding (Trenbath and Harper 1974, Harper 1977, Vandermeer 1989) in which two parts of a system improve their performance by interacting with each other. Using mildew infection as a covariate in the statistical analysis explained most of the differences in performance between plots with different GM richness or concentration, indicating that overall it was indeed the increased mildew resistance that caused the positive effects of GM richness and concentration on performance.

Differences among GM lines

Our experiment allowed us to test whether the introduction of different alleles of a *Pm3* transgene also affected plant performance. This was indeed the case. Even though the trait directly linked to the transgene, mildew resistance, was similar in both tested lines, we found that the phenological state and the start of flowering differed strongly between the two GM lines. Although at plot level biomass and seed yield did not differ, individual *Pm3a* plants had higher biomass and marginally higher individual seed production than *Pm3b*. The TSW analysis revealed that *Pm3a* had generally smaller seeds than *Pm3b*. It appears that the slower development of *Pm3a* allowed the individual plants to stay longer in the vegetative phase, develop more biomass and produce more but smaller seeds. Since both GM lines had similar mildew resistance, it is not likely that the performance differences described above were caused directly by the powdery mildew infections or allelic differences between the two lines. Since the lines differed both in the identity of the allele and the transformation event, it is conceivable that their different performance was due to effects related to the latter, e.g.

different gene expression levels as a consequence of different location of the insertion site (Cubas *et al.* 1999, Filipecki and Malepszy 2006). Such expression differences were for example observed in a previous study using multiple transformation events with a single *Pm3* allele (Zeller *et al.* 2010).

Individual plant- or plot-level effects of diversity? (H3, H4)

An understanding of the mechanism by which diversity affects plant yields at the plot or field level requires an assessment of effects on individual plants, in addition to an assessment of plot-level performance. For example, determining that higher yields in more diverse plots result from increases in plant density rather than increases in individual plant yield requires measurements of yield at both the individual and plot levels. Our analyses allow us to distinguish between a density effect of transgene diversity (H3) and an effect of transgene diversity on individual plant performance (H4). The similarity of results of statistical analyses at the individual plant and plot level support H4 (Tables S1 and S2 in Supplemental Material). Differences in phenological development and TSW among the two GM lines were found with both methods. GM richness and GM concentration showed similar trends for biomass, seed yield and TSW. Only the significantly increased seed yield due to increased GM richness at the plot level would not have been predicted by the results from individual plants. The explanation might lie in the density dependence of seed yield. Individual plants can and should be used for all traits like plant height, phenological development, TSW and seed set. However, for correct estimates of biomass and seed yield, the crop density or number of tillers would have to be included in the extrapolation from individual plant to the whole plot.

Generally, assessment of individual plants proved to be useful in testing the performance of genetically modified wheat lines. This method might be labor intensive but there are also several advantages: only a few plants need to be removed from each plot. This means that the experimental plots stay intact and can be used for other purposes. Furthermore, individual plants can be handled and stored much easier than bulky harvest bags. An important caveat, which must be considered in each case, is a potential confounding of plant density with treatment effects.

Conclusions

Our study demonstrated that mixing wheat lines that differed only in their resistance to different strains of powdery mildew reduced plant susceptibility to this pathogen. This led to an increased performance of these mixtures and even to transgressive overyielding. Not only mixtures of two GM lines compared to monocultures of one GM line, but also mixtures of one GM and one control line compared to monocultures of GM and control lines showed increased mildew resistance and in most cases also higher performance. One could therefore argue that mixing closely related plant lines could increase agricultural output. Ecological research indicates that productivity increases with diversity in most cases that have been experimentally investigated (Tilman *et al.* 1996, Hector *et al.* 1999, Roscher *et al.* 2005, Marquard *et al.* 2009). However, these results have not been translated into agricultural practice, in part because mixtures of different varieties are difficult to harvest. Gene technology might provide us with very similar plant lines that differ only in their resistance genes. Such mixtures could therefore be harvested without change of practice. We have only assessed mixtures of two lines, either two GM lines or mixtures of one GM and one control line. According to ecological theory, mixtures of more than two lines should lead to even better results. In the future, results of such mixture experiments should be compared to lines that have several resistance genes stacked within the same plant. It may be that costs of resistance would accumulate in such plants, thus potentially diminishing the synergistic benefit of transgene mixtures at the plot level, but further study would be needed to evaluate this hypothesis.

Furthermore, the evolution of resistant pathogens should be studied. Some studies report that resistances may develop faster if single-gene plants that harbor different resistance genes are planted next to double-gene plants (Zhao *et al.* 2005). However, it is also possible that the resistance development is slower in mixtures due to the lower pathogen population size (Chin and Wolfe 1984).

The comparison of two GM lines that harbor a different allele of the *Pm3* gene revealed a number of phenotypic changes in performance-related traits which might have been of pleiotropic origin. Several studies report that genetically modified plants might differ in many traits even if they share very similar transgenes (Snow *et al.* 2005, Filipecki and Malepszy 2006).

Finally we checked whether results obtained from individual plants can help to predict the performance of entire populations. We conclude that such measurements can be very useful for performance tests — especially when information about the variation and interactions within the population are of interest. We conclude that today's agricultural systems might become both more productive and more sustainable with biodiversity strategies such as planting line mixtures.

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Figures

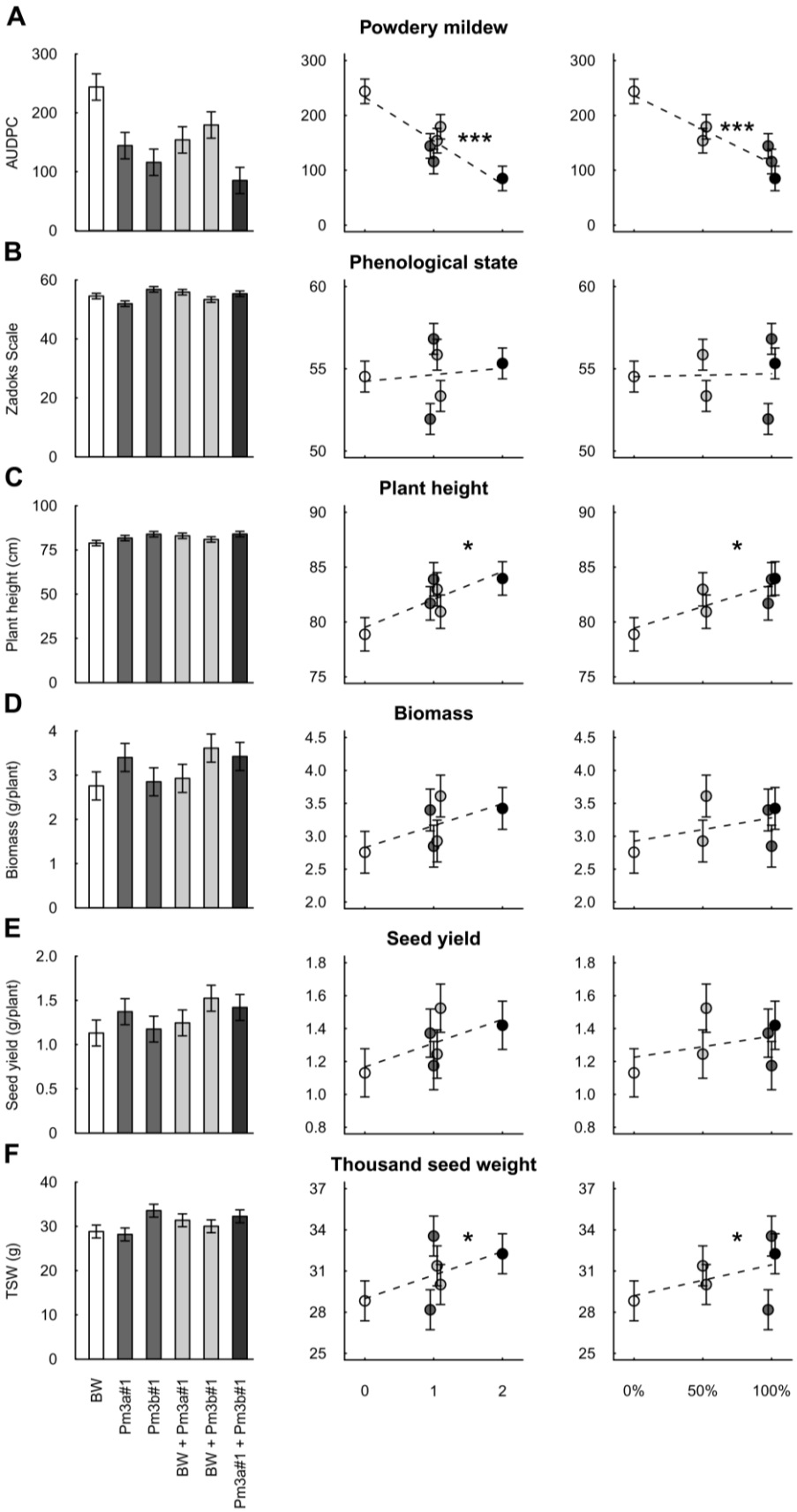
A
Powdery mildew
AUDPC
300
200
100
0
***
***
B
Phenological state
Zadoks Scale
60
40
20
0
55
50
C
Plant height
Plant height (cm)
100
75
50
25
0
90
85
80
75
*
*
D
Biomass
Biomass (g/plant)
4
3
2
1
0
4.5
4.0
3.5
3.0
2.5
2.0
E
Seed yield
Seed yield (g/plant)
2.0
1.5
1.0
0.5
0
1.8
1.6
1.4
1.2
1.0
0.8
F
Thousand seed weight
TSW (g)
40
30
20
10
0
37
34
31
28
25
*
*
BW
Pm3a#1
Pm3b#1
BW + Pm3a#1
BW + Pm3b#1
Pm3a#1 + Pm3b#1
0
1
2
0%
50%
100%

Figure 1. Effects of GM richness and GM concentration on individual wheat plants. Line means were predicted using REML models. GM richness consisted of the levels “no GM” “one GM” and “two GM” lines and GM concentration of 0, 50 and 100% GM plants in a particular plot. A–F are different traits that were measured on individual plants. Asterisks indicate the level of significance for the GM richness or GM concentration contrast (*P<0.05; ***P<0.001).

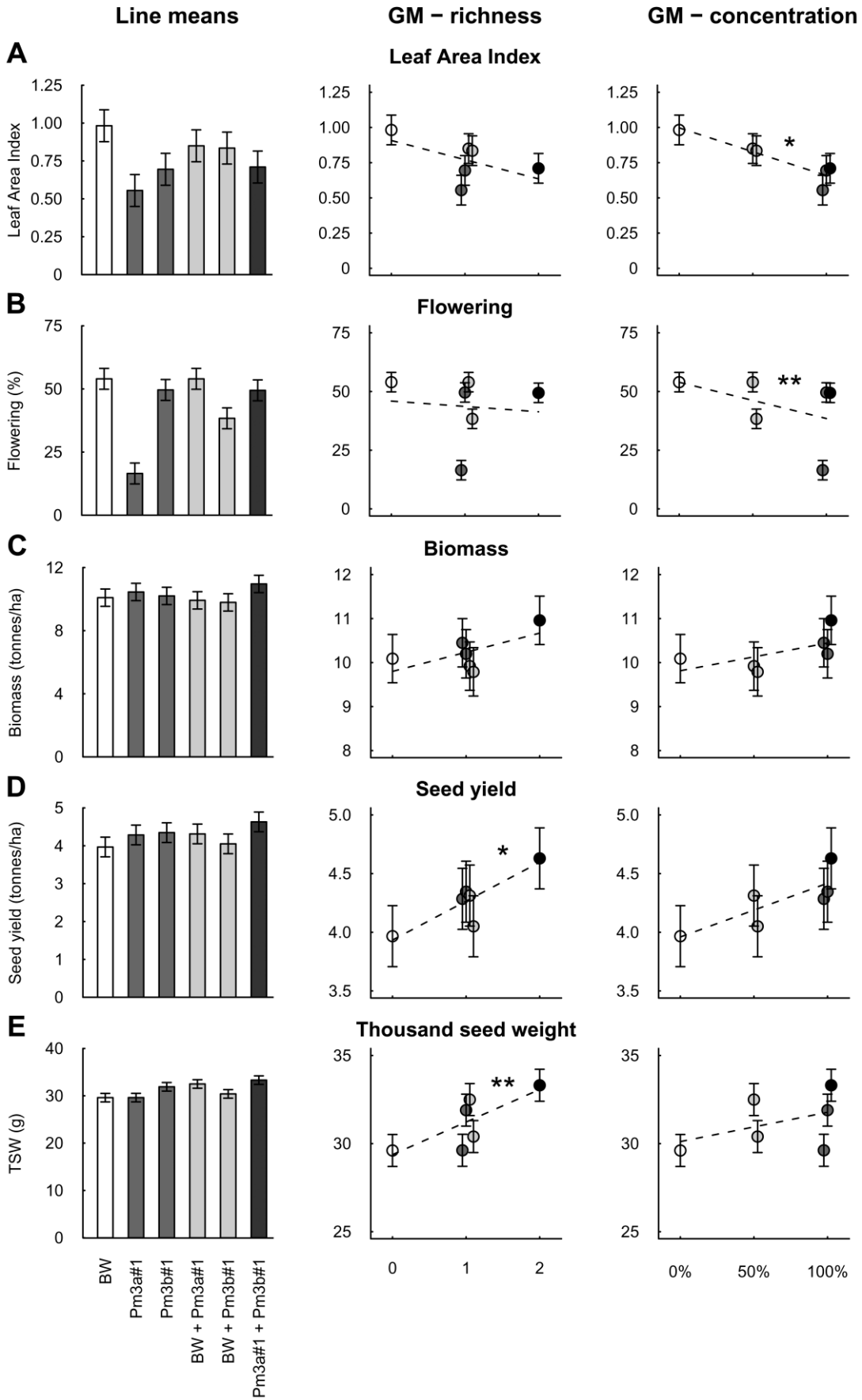
Line means
GM − richness
GM − concentration
A
Leaf Area Index
Leaf Area Index
B
Flowering (%)
Flowering
C
Biomass (tonnes/ha)
Biomass
D
Seed yield (tonnes/ha)
Seed yield
E
TSW (g)
Thousand seed weight
*
**
BW
Pm3a#1
Pm3b#1
BW + Pm3a#1
BW + Pm3b#1
Pm3a#1 + Pm3b#1
0
1
2
0%
50%
100%

Figure 2. Effects of GM richness and GM concentration on wheat at the plot level. Line means were predicted using REML models. GM richness consisted of the levels "no GM" "one GM" and "two GM" lines and GM concentration of 0, 50 and 100% GM plants in a particular plot. A–E are different traits that were measured at the plot level. Asterisk indicate the level of significance for the GM richness or GM concentration contrast (*P<0.05; **P<0.01).

Supplemental Material to Chapter 4

Table S1. Summary of Manova and REML tables of the traits measured on individual plants. Three different models were used. In Model 1 all treatments were pooled. In Model 2 GM richness was fitted first and confounding effects of GM concentration were ignored. In Model 3, GM richness is fitted second, measuring the difference between richness levels corrected for increasing GM concentration. Random terms are not included in the table since their variance components are estimated directly in the REML analyses. The percentage Rao's F-statistics or Wald statistic were thus calculated only for the total of the fixed effects.

			Manova		AUDPC		Phenological state		Plant Height		Biomass		Seed Yield		Thousand seed weight	
Model	Source of variation	df	% RaoF	F pr.	% Wald	F pr.	% Wald	F pr.	% Wald	F pr.	% Wald	F pr.	% Wald	F pr.	% Wald	F pr.
1	Treatment	5	100	<0.001	100	<0.001	100	0.006	100	0.087	100	0.176	100	0.176	100	0.009
2	GM richness	1	36.4	0.002	83.9	<0.001	2.0	0.512	67.1	0.013	34.6	0.099	36.3	0.092	27.7	0.022
2	GM concentration	1	6.7	0.277	11.2	0.031	1.2	0.756	8.5	0.328	0.7	0.814	3.6	0.581	0.4	0.770
3	GM concentration	1	37.2	0.002	84.9	<0.001	0.2	0.754	67.2	0.013	16.3	0.245	12.2	0.314	19.9	0.047
3	GM richness	1	5.9	0.341	10.3	0.038	3.0	0.513	8.4	0.330	18.8	0.215	27.8	0.136	8.1	0.187
2+3	GM richness × GM concentration	1	2.3	0.778	0.0	0.902	0.7	0.478	1.4	0.690	4.9	0.515	10.2	0.356	0.1	0.876
2+3	*Pm3a* vs. *Pm2b*	1	44.7	0.001	4.8	0.141	87.2	0.001	23.0	0.118	59.2	0.036	48.5	0.055	52.2	0.003
2+3	GM concentration × *Pm3a*	1	9.9	0.131	0.0	0.931	8.9	0.1	0.0	0.955	0.7	0.804	1.5	0.724	18.7	0.053
									1 value excluded							

Table S2. Summary of Manova and REML tables of the traits measured at the plot level. Three different models were used. In Model 1 all treatments were pooled. In Model 2 GM richness was fitted first and confounding effects of GM concentration were ignored. In Model 3, GM richness is fitted second, measuring the difference between richness levels corrected for increasing GM concentration. Random terms are not included in the table since their variance components are estimated directly in the REML analyses. The percentage of Rao's F-statistics or Wald statistic were thus calculated only for the total of the fixed effects.

			Manova		LAI		Flowering		Biomass		Seed Yield		Thousand seed weight	
Model	Source of variation	df	% RaoF	F pr.	% Wald	F pr.	% Wald	F pr.	% Wald	F pr.	% Wald	F pr.	% Wald	F pr.
1	Treatment	5	100	0.002	100	0.021	100	<0.001	100	0.516	100	0.327	100	0.021
2	GM richness	1	9.6	0.131	33.0	0.085	1.1	0.466	43.2	0.190	79.4	0.040	54.6	0.006
2	GM concentration	1	28.3	0.005	57.3	0.028	31.9	<0.001	2.3	0.736	3.2	0.685	5.5	0.340
3	GM concentration	1	18.9	0.021	85.4	0.010	19.3	0.005	36.4	0.220	63.5	0.064	18.7	0.081
3	GM richness	1	18.9	0.020	4.9	0.488	14.2	0.012	9.1	0.556	19.0	0.289	42.2	0.013
2+3	GM richness × GM percentage	1	5.4	0.360	0.0	0.972	3.0	0.216	50.0	0.157	4.8	0.586	0.0	0.987
2+3	*Pm3a* vs. *Pm2b*	1	51.6	0.000	5.8	0.470	56.8	<0.001	0.0	0.884	9.5	0.451	39.9	0.016
2+3	GM percentage × *Pm3a*	1	5.2	0.376	3.9	0.559	7.3	0.060	4.5	0.679	3.2	0.639	0.0	0.915

ang transformed

CHAPTER 5

Costs of Resistance to Fungal Pathogens in Genetically Modified Wheat

Simon L. Zeller, Olena Kalinina, Bernhard Schmid. 2012. Journal of Plant Ecology. DOI 10.1093/jpe/rts013

Seed set of a single spike of Bobwhite (left) and transgenic *Pm3b*#2 line (right)

Abstract

Many resistance genes against fungal pathogens show costs of resistance. Genetically modified (GM) plants that differ in only one or a few resistance genes from control plants present ideal systems for measuring these costs in the absence of pathogens. To assess the ecological relevance of costs of pathogen resistance, we grew individual plants of four transgenic spring wheat lines in a field trial with three pathogen levels and varied the genetic diversity of the crop.

We found that two lines with a *Pm3b* transgene were more resistant to powdery mildew than their sister lines of the variety Bobwhite whereas lines with *chitinase* or *chitinase* and *glucanase* transgenes were not more resistant than their mother variety Frisal. Nevertheless, in the absence of the pathogen, both the GM lines of Bobwhite as well as those of Frisal performed significantly worse than their controls, i.e. *Pm3b*#1 and *Pm3b*#2 had 39% or 53% and A9 *Chi* and A13 *Chi/Glu* had 14% or 23% lower yields. In the presence of the pathogen, all GM lines except *Pm3b*#2 could increase their yields and other fitness-related traits, reaching the performance levels of the control lines. Line *Pm3b*#2 seemed to have lost its phenotypic plasticity and had low performance in all environments. This may have been caused by very high transgene expression. No synergistic effects of mixing different GM lines with each other were detected. This might have been due to high transgene expression or the similarity between the lines regarding their resistance genes.

We conclude that costs of resistance can be high for transgenic plants with constitutive transgene expression that this can occur even in cases where the non-transgenic control lines are already relatively resistant, such as in our variety Frisal. Transgenic plants could only compete with conventional varieties in environments with high pathogen pressure. Furthermore, the large variability among the GM lines, which may be due to unpredictable transgene expression, suggests that case-by-case assessments are necessary to evaluate costs of resistance.

Introduction

Plants interact with their environment in various ways. They have to compete with their neighbors and endure abiotic stresses and pathogen attacks. Natural selection can improve competitiveness and stress resistance. However, there are no wild plants with resistances against all possible pathogens (Bergelson and Purrington 1996a), an observation consistent with the idea of a trade-off between performance and defence (Herms and Mattson 1992). Genes that increase resistance against pathogens may be costly for a plant in the absence of pathogens. A meta-analysis showed that resistant plants had lower fitness than non-resistant ones in approximately half of 88 studies considered (Bergelson and Purrington 1996a). It is important to understand the mechanism leading to such costs and how these affect plant–pathogen systems, as such knowledge is relevant for basic ecology as well as for agricultural ecosystems (Brown 2002).

Fitness costs that are associated with pathogen resistance are difficult to measure. Resistance genes are often linked to other, making it difficult to elucidate single-gene costs of resistance. This problem can be avoided by using transgenic (genetically modified = GM) plants that differ only in one or a few known genes from their original genetic background (Burdon and Thrall 2003, Purrington 2000). Thus, transgenic crop plants may serve as model systems for ecologists interested in costs of pathogen resistance, even though they may differ in some aspects from wild plants.

Few studies to date have measured costs of resistance in transgenic plants (Burdon and Thrall 2003, Bergelson *et al.* 1996b, Purrington 2000, Romeis *et al.* 2007, Tian *et al.* 2003, Vila-Aiub *et al.* 2009). Resistance costs of transgenes have been found in some but not all of these studies (Snow *et al.* 1999). Even if such costs exist, they have to be put into the right context. There are very few studies (e.g, Brunner *et al.* 2011) that varied the pathogen pressure, which is necessary to study the ecological relevance of costs associated with resistance genes. The pathogen level can itself be influenced by the plant community which can either facilitate or slow down the spread of epidemics. In particular, genetic diversity for pathogen resistance in a plant stand can reduce the pathogen pressure and therefore increase the performance at the level of the population and of individual plants (Mundt 2002, Schmid 1994, Wolfe 2000). However, we did not find any published reports where the influence of pathogen pressure and community diversity on plant performance and costs of resistance were evaluated in combination.

We therefore performed a field trial with four transgenic and two non-transgenic lines of spring wheat *Triticum aestivum* L. that belonged either to the variety Bobwhite or Frisal. The GM Bobwhite lines *Pm3b*#1 and *Pm3b*#2 harboured a *Pm3b* transgene against powdery mildew *Blumeria graminis* f.sp. *tritici* (DC.) Speer, whereas the Frisal lines A9 *Chi* and A13 *Chi/Glu* had either a *chitinase* or a *chitinase* and a *glucanase* gene, respectively, to induce quantitative fungal resistance. These transgenic lines were produced from commercially available Bobwhite or Frisal plants which we took as controls. We established three fungal infection treatments. One third of the studied plants were sprayed with fungicide to prevent powdery mildew infection, to allow measurement of potential costs of resistance in the absence of the pathogen. Furthermore, plants were naturally or artificially infected with powdery mildew to obtain different pathogen infection levels. We worked with individual plants that were hand-seeded into plots containing either Bobwhite or Frisal lines of varying genetic diversity (0, 1 or 2 GM lines). The factorial design, combining the different wheat lines with fungal infection and genetic diversity treatments, allowed us to address the following questions: (i) are there differences between GM and non-GM lines and between different GM lines? (ii) are there costs of resistance in the absence of pathogens? And (iii) does the mixing of plant lines and therefore the increase of genetic diversity increase resistance and performance and are there interactions between fungal infection and diversity treatments?

Materials and Methods

Genetically modified wheat

We used six spring wheat lines of the Mexican variety Bobwhite SH 98 26 (Brunner *et al.* 2011, Lindfeld *et al.* 2011, Peter *et al.* 2010, von Burg *et al.* 2010, von Burg *et al.* 2011, Zeller *et al.* 2010) and the Swiss variety Frisal (Bieri *et al.* 2003, Kalinina *et al.* 2011) for our experiment. Two GM and one non-GM line were chosen from each variety.

The GM lines of Bobwhite harboured a *Pm3b* transgene in different position on the genome, each derived from different transformation events. *Pm3b* confers race-specific resistance to powdery mildew and was obtained from the hexaploid wheat variety Chul (Yahiaoui *et al.* 2004). The lines, which were named *Pm3b#1* and *Pm3b#2,* were generated by biolistic transformation (Pellegrineschi *et al.* 2002). The plasmids pAHC17+NotI (*PMI*) and pAHC17+3NotI (*Pm3b*) were used as vectors

(Christensen and Quail 1996; Travella *et al.* 2006). After *Not*I (for *Pm3b*) or *Not*I/*Hin*dIII (for *PMI*) digestion, only the desired fragments, but no vector sequences, were co-bombarded into wheat. The *Pm3b* gene was cloned under the control of the *Zea mays* L. (maize) ubiquitin promoter (Christensen and Quail 1996). More detailed information can be found in previous studies (Zeller et al. 2010, Brunner et al. 2011). Presence of the transgenes was confirmed by Southern hybridization analysis (Southern 2006). The GM lines contained the *Pmi* gene as well as one complete copy of *Pm3b*, which segregated as a single Mendelian locus in the T_1 generation. Two *Pm3b* lines were multiplied to T5 and used for the field experiment. The level of transgene expression was assessed by quantitative real time PCR using RNA isolated from leaves of field-grown plants. It revealed that *Pm3b* genes in the lines *Pm3b*#1 and *Pm3b*#2 were expressed constitutively and that the mean expression level was 11 and 55 times higher than in the variety Chul, where this gene is expressed naturally (Brunner *et al.* 2011; Zeller *et al.* 2010).

The two transgenic lines with the genetic background of the variety Frisal contained genes from barley which are known for their anti-fungal effect and the constitutive or inducible expression of pathogenesis-related genes (Zhu *et al.* 1994). Line A9 *Chi* harboured a *chitinase* and A13 *Chi/Glu* both a *chitinase* and a β-1,3-*glucanase* transgene (Bliffeld *et al.* 1999). Both lines were generated by biolistic transformation (Pellegrineschi *et al.* 2002). A maize ubiquitin promoter (Christensen and Quail 1996) was used for the *chitinase* and an actin promoter from rice (McElroy *et al.* 1990) for the β-1,3-*glucanase*. The expression of the transgenes *chitinase* and β-1,3-*glucanase* was analyzed by SDS-PAGE and Western blotting of intercellular wash fluid from mature leaves, and in later generations on total protein from seedling leaves (Bieri et al. 2003). Both lines were multiplied to T6 in the glasshouse in order to verify stable expression of the transgenes.

Field experiment

The field experiment took place at an agricultural research station in Zurich-Reckenholz, Switzerland, at 440 m above sea level. It started in March 2009 and lasted until beginning of August 2009. Three powdery-mildew treatment blocks, each with twelve 1.0 x 1.3 m plots, were sown with seeds of the six lines described above (Figure S1 in Supplemental Material). Besides the monocultures, six plots with 50:50 mixtures consisting of *Pm3b*#1/Bobwhite control, *Pm3b*#2/Bobwhite control, *Pm3b*#1/*Pm3b*#2

as well as A9 *Chi*/Frisal control, A13 *Chi/Glu*/Frisal control, A9 *Chi*/A13 *Chi/Glu* were sown to assess mixture effects. In each plot five rows with a distance of 20 cm between them were sown at a density of 400 seeds per m^2 using a Seedmatic system (Hege 90, Hege Maschinen, Eging am See, Germany). To assess the performance of individual plants it was essential to know the line identity of plants in mixture plots. We therefore inserted short sections consisting of 7 seeds ("seed islands") of known identity by hand into the prepared rows. This was done right after the machine sowing. Each island was shifted slightly relative to the machine-sown row to allow the removal of machine-sown seedlings immediately after emergence (see Figure S1 in Supplemental Material). Monocultures received one and mixture plots two islands. This planting procedure guaranteed that the hand-sown seeds in these seed islands had an almost identical competitive environment as the machine-sown seeds. Three out of the seven planted seedlings per island (position 2, 4, 6) were marked with a label after emergence.

The three fungal infection treatments were fungicide application and natural and artificial mildew infection. Fungicide plots were sprayed three times with the fungicide Prosper (500g l^{-1} Spiroxamine; Leu + Gygax AG, Birmenstorf, Switzerland). This allowed keeping the plots almost completely free of powdery mildew. In the natural infection plots, neither artificial inoculation nor fungicides were applied. All untreated plots were infected strongly by powdery mildew during the field experiment. The plots with artificial powdery mildew infection were bordered with "spreader rows" of the susceptible conventional winter wheat variety Kanzler. The plants of the spreader rows had been pre-grown and inoculated with powdery mildew, isolate 96224, in the glasshouse. The distance between spreader rows and plots was 80 cm. The powdery mildew isolate 96224 had been collected between Winterthur and Kloten (Switzerland) in 1996 (Brunner *et al.* 2010; Srichumpa *et al.* 2005) and was known to be avirulent on *Pm3b* (Yahiaoui *et al.* 2009). A second batch of inoculated plantlets were produced and planted one month later. The three fungal infection treatments were separated from each other by a 4-m wide border crop of spring triticale to reduce cross-contamination.

Based on a nutrient assessment different amounts of nitrogen fertilizer were applied before sowing. This resulted in equal nitrogen concentrations (7.5g N m^{-2}) in each block. At the phenological stages 22–29 (Zadoks *et al.* 1974) additional nitrogen was added (3 g N m^{-2} as "Ammonsalpeter 27.5", Lonza, Visp, Switzerland). The natural field soil provided the plants with sufficient phosphorous, potassium and magnesium (81, 176 and 248 mg kg^{-1}). All plots were sprayed with the herbicide cocktail Concert

SX (40% Thifensulfurone, 4% Metusulfurone-methyl; Stähler Suisse AG, Zofingen, Switzerland) and Starane super (120 g l^{-1} Bromoxynil, 120 g l^{-1} Ioxynil, 100 g l^{-1} Fluroxypyr-metilheptil-ester; Omya Agro AG, Safenwil, Switzerland) at the beginning of May. Insecticide Karate Zeon (100g l^{-1} Lambda-Cyhalothrin; Syngenta Agro AG, Dielsdorf, Switzerland) against the wheat stem fly (*Chlorops pumilionis* Bjerk.) was applied at the beginning of May and repeated 2 weeks later.

Response variables

The degree of powdery mildew infection (Eyal *et al.* 1987) was assessed 32, 45, 59 and 80 days after germination. Based on these data, we calculated the "Area Under Disease Progress Curve", AUDPC (Jeger and Viljanen-Rollinson 2001). AUDPC is the amount of disease integrated over the time period of interests. It is based on the trapezoidal rule for calculating areas (Jeger and Viljanen-Rollinson 2001). After ripening, all marked plants were cut at ground level and separated into vegetative and reproductive parts (spikes). Vegetative and reproductive parts were then dried at 80 and 25 C°, respectively, and weighed. We then threshed the reproductive parts and obtained the seed mass which is equivalent to seed yield. The seeds obtained from all spikes of a plant were counted by hand. Vegetative mass was calculated by subtracting the seed mass from the total biomass. Furthermore, plant height was measured at the highest point of the plant from the soil, 80 days after germination.

Data analysis

We analyzed the data with mixed-model analysis of variance using the classical ANOVA as well as the REML (Restricted Maximum Likelihood) method with the statistical software GenStat (VSN International Ldt). Results were almost identical and thus only the REML analyses are presented in this paper because they are considered to yield better results when missing values occur in a data set (Payne et al. 2010). In contrast to the classical method, which fits a mean for each level of a random-effects term, the REML method directly estimates the variance components of such terms. We used blocks, the block x fungal treatment interaction, plots nested within this interaction and islands nested within plots as random-effects terms in the analysis. Using these random-effects terms and the REML approach ensured that fixed-effects terms were automatically tested against appropriate error terms (Payne et al. 2010). Terms for fixed effects were fitted with hierarchical and factorial models as follows.

First, we used an "all hierarchical" treatment/line model that sequentially i) divided the line effects (six levels) into a contrast between Bobwhite and Frisal plants, ii) added within each variety the fungal infection treatment (three levels) as a iia) contrast between fungicide and mildew infection and a iib) contrast between natural and artificial infection within the latter, iii) added the two remaining line-effects contrasts iiia) control vs. GM lines and iiib) differences between the two GM lines within each variety (Model 1; Figure 1, Table S1 in Supplemental Material). Second, we used a "factorial sub-model" after the initial contrast i) between Bobwhite and Frisal for each of the two varieties separately. The sub-model contained the main effects of fungal infection treatment, divided into the two contrasts iia) and iib), the main effects of the two remaining line-effects contrasts iiia) and iiib) and the corresponding four contrast interactions (Model 2; Table S2 in Supplemental Material). The advantage of these contrast formations was that they yielded focused single-degree of freedom tests (Rosenthal and Rosnow 1985). As recommended by these authors, we used this approach of focused comparison instead of post-hoc multiple comparison tests.

Two additional terms were added to these two models to assess the influence on the target plants of the number of GM-lines (GM richness 0, 1 and 2) or the proportion of GM-plants (GM concentration 0, 50, 100%) per plot. Since these two contrasts were partly confounded with each other, their fitting sequence was alternated in two separate runs of the analyses. Furthermore, these contrasts were either fitted before or after the effects of the lines and the fungal infection treatment. Fitting GM richness and -concentration first in the models allowed an assessment of their influence "ignoring" confounding effects of the lines (effects of fungal infection treatment were not confounded with GM richness or GM concentration and therefore in this case the fitting sequence did not matter). Fitting GM richness and concentration after the fungal infection treatment and line effects allowed an assessment of their influence "eliminating" confounding effects of the lines (see e.g. McCullagh and Nelder 1989 for the ignoring/eliminating terminology).

To understand better the effects of fungal infection treatments and GM richness and GM concentration within each, Bobwhite or Frisal, we repeated all analyses with datasets restricted to either of the two varieties. However, we mostly present results from the full model.

Residual plots were examined to check if the assumptions of normality and homoscedasticity were fulfilled. Seed yield, vegetative mass and seed number were

square-root transformed and x^2 transformation was necessary for phenological state, plant height, spike length and TSW. Back-transformed means and standard errors from the REML output were used to draw the figures. The critical significance level was 0.05 in all analyses.

Since several of the measured traits correlated with each other, we also performed a Multivariate Linear Mixed Model (MLMM) to test for the overall significance of fungal infection treatment and line effects. The five traits AUDPC, plant height, seed yield, vegetative mass and seed number were combined in a single analysis. Transformed data were used for the MLMM analysis.

Results

Powdery mildew infection

The spring wheat variety Bobwhite was more susceptible to powdery mildew than the old Swiss variety Frisal ("Bobwhite vs. Frisal": P<0.001; Figure 2a and Table S1 in Supplemental Material). The repeated spraying with fungicide reduced mildew infections by a factor of 6.2 for Bobwhite and by a factor of 5.4 for Frisal plants ("Fungicide vs. Mildew" within Bobwhite or within Frisal both P<0.001, see Table S1 in Supplemental Material). The natural and artificial mildew treatment levels did not differ significantly from each other with regard to mildew infection, both within Bobwhite or within Frisal. Nevertheless, we assume that the composition of the pathogen community differed between these two treatment levels because of the artificial infection was done with only one particular powdery mildew strain. The Bobwhite GM lines *Pm3b*#1 and *Pm3b*#2 were less susceptible to powdery mildew than the non-transgenic Bobwhite control line in all three fungal infection treatments (83, 52 and 61% less mildew in fungicide-treated, natural infection and artificial infection plots, respectively). *Pm3b*#2 had 36% less powdery mildew than *Pm3b*#1 in the plots with natural infection (P<0.001; "*Pm3b*#1/2 in Natural in Table S1 in Supplemental Material). There was no such difference between the two Bobwhite GM lines in the plots with artificial infection where a mildew strain avirulent for *Pm3b* genes was released.

Mildew infections decreased with increasing GM concentration in the plots (GM concentration fitted before line effects: P<0.001, data not shown). Results for GM richness were less clear. GM-rich plots had significantly less mildew when GM richness was fitted before GM concentration. However, this signal was lost when GM

concentration was fitted before GM richness. To understand why GM concentration and GM richness reduced the mildew infection levels in diverse plots, we performed further analyses. We fitted GM concentration and GM richness after fungal infection treatment and line effects and interactions and therefore eliminated these (see Material and Methods). As a result, the significant results from above disappeared (see Table S1 in Supplemental Material), which means that the decreased powdery mildew infection can be explained by the different pathogen resistance levels of the individual lines (line effects). The GM-Frisal lines A9 *Chi* and A13 *Chi/Glu* showed no increased pathogen resistance when compared to plants of the Frisal control line and also no differences for GM concentration or GM richness. The mixing of lines *Pm3b*#1 with *Pm3b*#2 or A9 *Chi* with A13 *Chi/Glu* did therefore not lead to synergistic reduction of powdery mildew infection levels.

Fungal infection treatment effects and differences between GM and control lines in these (all hierarchical model)

Plants of the variety Bobwhite differed from Frisal in all traits (MLMM, "Bobwhite vs. Frisal": $P<0.001$). The performance of Bobwhite and Frisal plants depended strongly on the fungicide or mildew treatment levels and therefore on the pathogen pressure (MLMM, "Fungicide/Mildew" for Bobwhite and Frisal both with $P<0.001$). Neither Bobwhite nor Frisal lines performed differently in plots with natural as compared with artificial infection. We describe the Bobwhite results first, followed by Frisal.

The fungicide application increased plant height within the Bobwhite variety ($P=0.002$; "Fungicide vs. Mildew in Bobwhite" for plant height in Table S1 in Supplemental Material). However, there were no overall positive effects on seed yield or vegetative mass because of line-specific responses to the fungicide application. Seed yields of plants of the Bobwhite control line and the GM line *Pm3b*#2 were 31% and 13% higher, respectively, under fungicide application, whereas they were 28% lower for plants of the GM line *Pm3b*#1.

Bobwhite GM lines reacted differently to fungicide spraying compared to Bobwhite control lines ($P=0.005$; "Fungicide/Mildew × BW/GM within variety Bobwhite" for seed yield in Table S2 in Supplemental Material). When comparing the Bobwhite control with the mean of the two Bobwhite GM lines in the fungicide-treated plots , we found that the latter had 42% fewer seeds ($P<0.001$), 46% lower seed yield ($P<0.001$), 34% lower vegetative mass ($P<0.001$) and 7% lower plant height ($P=0.002$;

"BW/GM in Fungicide" in Table S1 in Supplemental Material). The seed yield of line *Pm3b*#1 was 39% and that of line *Pm3b*#2 was 53% lower when compared to Bobwhite control. These results indicate that the Bobwhite GM lines, in contrast to the control line, did not benefit from the absence of the pathogens. Bobwhite GM lines had on average less seeds than Bobwhite control in the natural infection treatment level (P=0.016; "BW/GM in Natural" for seed number in Table S1 in Supplemental Material).

Frisal lines that were sprayed with fungicide grew taller than unsprayed plants ("Fungicide vs. Mildew within Frisal" for plant height: P=0.003; Table S1 in Supplemental Material). As for the Bobwhite lines, the two Frisal GM lines had on average 20% fewer seeds (P=0.026), 18% lower yield (P=0.043) and 6% lower plant height (P<0.001) than the control line ("Frisal/GM in Fungicide"; Table S1 in Supplemental Material) in the sprayed plots. We found that the yield of line A9 *Chi* was 14% and that of line A13 *Chi/Glu* was 23% lower when compared to Frisal control. No such differences were found for plants growing in plots with natural or artificial infection.

Differences between GM-lines (factorial submodel)

Although the two GM lines of Bobwhite, *Pm3b*#1 and *Pm3b*#2, had the same transgene, they had very different phenotypes (MLMM, "*Pm3b*#1/2": P<0.001). *Pm3b*#2 had 19% fewer seeds (P=0.051), 41% lower seed yield (P<0.001), 19% lower vegetative mass (P=0.058) and a 5% reduced height (P<0.001) compared with *Pm3b*#1 ("*Pm3b*#1/2"; Table S2 in Supplemental Material). In addition to this overall difference, the two GM lines also showed different responses to the two mildew treatments levels ("Fungicide/Mildew × *Pm3b*#1/2" for vegetative mass: P=0.038; Table S2 in Supplemental Material). This was due to a higher relative performance of *Pm3b*#1 in plots with mildew than with fungicide whereas no such response was found for line *Pm3b*#2. However, even the GM line *Pm3b*#1 never reached the performance of control plants in fungicide plots. The yield of unsprayed *Pm3b*#1 was 21% and that of *Pm3b*#2 59% lower than that of the Bobwhite control line in the fungicide treatment level.

Also in the variety Frisal the two GM lines, A9 *Chi* and A13 *Chi/Glu*, had different phenotypes (MLMM, "A9/A13": P<0.001). Plants of line A9 *Chi* were 4% shorter (P<0.001) and had 18% more seeds (P=0.015) than A13 *Chi/Glu* ("A9/A13";

Table S2 in Supplemental Material). As for the Bobwhite GM lines, also the Frisal GM lines could never reach the yields of sprayed Frisal control plants. Unsprayed A9 *Chi* plants had 20% and unsprayed A13 *Chi/Glu* plants had 27% lower seed yields than sprayed plants of the Frisal control line.

Effects of GM concentration and GM richness

The genetic diversity of the plot into which the tested plants were sown influenced their performance. Plants in plots with higher GM concentration had fewer seeds ($P<0.001$), lower seed yield ($P=.005$) and were shorter ($P<0.001$) than plants in plots with higher GM concentration. To understand why GM concentration had mostly negative effects on fitness-related traits, we fitted GM concentration and GM richness after line and fungal infection treatment effects and interactions and therefore eliminated these (see Materials and Methods). As a result, all significant results from above disappeared (see Tables S1 and S2 in Supplemental Material). By looking at the data we could see that the good performance of Bobwhite control and the bad performance of line *Pm3b*#2 underlie most of the concentration and richness effects. No synergistic effects caused by the mixing of lines *Pm3b*#1 with *Pm3b*#2 or A9 *Chi* with A13 *Chi/Glu* were detected.

Discussion

Powdery mildew infection

Our results show that the two tested spring wheat varieties differed from each other. Bobwhite lines proved to be more susceptible to powdery mildew than the Swiss variety Frisal. This might have to do with different breeding aims and the origin of these varieties. In Switzerland, where powdery mildew is a serious plant disease, breeders have favored resistant varieties whereas this was not necessary in Mexico where no natural epidemics occur (Lillemo *et al.* 2006). Frisal entered the official variety list of Switzerland in 1987. After the release, the susceptibility to powdery mildew and leaf rust increased during the nineties (M. Winzeler, personal communication). Frisal was subsequently taken off the market in 2006. It is therefore not surprising that not only Bobwhite but also Frisal lines were infected by this pathogen. The GM lines *Pm3b*#1 and *Pm3b*#2 proved to be more resistant to powdery mildew than their genetic background Bobwhite. No such differences were detected in the A9 *Chi* and A13 *Chi/Glu* lines which were produced from Frisal. This may be

because Frisal control lines were already relatively resistant to powdery mildew. It is conceivable that this native resistance could not be improved by additional resistance genes. This result, however, contrasts with laboratory results where A9 *Chi* was less susceptible to powdery mildew than Frisal (Bieri *et al.* 2003). Hence, these results demonstrate the importance of field trials.

Since we worked in a natural environment it was not possible to remove the omnipresent natural mildew spores. However, the fungicide used in the fungicide treatment level reduced powdery mildew infections in all plots to almost zero. This allowed us to assess the influence of the pathogen pressure on fitness-related traits and unintended effects. The difference between the natural and artificial treatment levels was less prominent. There was no overall difference in pathogen abundance (AUDPC) between these two treatment levels, although the artificial infection started before the natural infection (data not shown). It is conceivable that climatic conditions and not the start of the inoculation mainly affected the spread and growth of powdery mildew. However, it is likely that the artificially introduced mildew isolate 96224 was more common in artificial than in natural infection plots. This strain is avirulent for (i.e. cannot attack) the two Bobwhite GM lines *Pm3b*#1 and *Pm3b*#2. We therefore expected less mildew in these plots than in the naturally infected ones. Indeed, line *Pm3b*#1 proved to be more resistant in the artificially than in the naturally inoculated plots. Line *Pm3b*#2, however, was highly resistant in both and this could have been due to the very high transgene expression levels of this line that made it even resistant to a "non-target" powdery mildew strain. Brunner *et al.* (2011) argued that high expression does provide some degree of quantitative resistance against different strains of powdery mildew.

Besides the mildew treatment levels, we analyzed the influence of plant diversity on individual plants within a plot. Plants in plots with high concentrations of resistant GM lines had less powdery mildew than plants in plots with the susceptible Bobwhite control line. This effect could be explained by the presence or absence of the susceptible Bobwhite line. One reason to include diversity treatments into our experimental design was to assess possible synergistic effects caused by the mixing of different GM lines. There are several publications that show improved pathogen resistance in fields with mixed varieties (Finckh *et al.* 2000; Mundt 2002; Wolfe 2000). However, we found no indications that mixed *Pm3b*#1 and *Pm3b*#2 plots were more resistant against powdery mildew than monocultures of these GM lines with identical

transgenes but different expression levels. There are at least two explanations for this. Either the influence of the mixed background was not strong enough to affect the plants which themselves belonged to uniform seed islands or these lines were too similar to allow synergistic or complementary effects. The same might be true for the Frisal lines. Although not genetically identical, all three Frisal lines were similarly resistant against powdery mildew in all three fungal infection treatments. Hence, in the absence of variability, no synergistic effects should perhaps have been expected.

Costs of resistance

If a transgene would induce complete pathogen resistance without any costs we would expect GM lines to perform as well as non-resistant control lines in absence of the pathogen. We found, however, that all four GM lines performed worse than their Bobwhite and Frisal control lines on fungicide-treated plots. In fact, none of the lines ever reached the level of the non-GM control lines even in the un-sprayed plots. This indicates that *Pm3b* as well as *chitinase* and *glucanase* transgenes cause costs of resistance. We found that the disadvantage of GM lines, as expected, decreased in plots with high pathogen levels.

Whereas costs of resistance might explain why these GM lines did not reach the level of the control lines in the absence of the pathogen, this does not explain why line *Pm3b*#1 performed worse in the fungicide than in the mildew treatment levels. One explanation could be that the chemicals of the fungicide interacted with the transgene or its products. Increased sensitivity to fungicide was described already earlier in a glasshouse study (Zeller *et al.* 2010). The sum of costs of resistance and fungicide sensitivity could have caused the large fitness reductions in lines *Pm3b*#1 and *Pm2b*#2. Since it is not possible to remove a common pathogen from a field without the use of pesticide one would have to revert to closed systems without pathogen presence to study costs of resistance separate from potential fungicide effects. However, costs of resistance might not be visible under conditions that are optimal for plant growth. A better approach then closed systems might be to carry out field trials in areas where the targeted pathogen does not occur naturally, or to stress the plants in the closed system.

Whereas line *Pm3b*#1 performed better in the mildew than in the fungicide treatment presumably due to benefits related to its powdery mildew resistance; *Pm3b*#2 performed poorly in all environments. For this line, costs of resistance seemed to be so high that potential benefits of the transgene were offset in all environments. Line

Pm3b#1 apparently could retain more plasticity than line *Pm3b*#2. This difference might be explained by the expression level. Line *Pm3b*#2 is known for much higher transgene expression levels than line *Pm3b*#1 (Brunner et al. 2011; Zeller et al. 2010). It is conceivable that costs of resistance increase with higher expression level because of increased metabolic stress. Besides the high expression levels, it would also be possible that not the gene dosage, but location-dependent interactions of the transgene with the native genome caused these negative effects (Bergelson *et al.* 1996b).

Among the GM Frisal lines, A13 *Chi/Glu* grew taller than A9 *Chi*. Seed yield and seed number were lower in line A13 *Chi/Glu* but these differences were not significant. We could therefore not prove that line A13 *Chi/Glu*, which harbours two transgenes, performs worse than line A9 *Chi* with only one. Further experiments are necessary to assess if the number of transgenes within a single plant increases costs of resistance.

GM plants with high costs of resistance may not be particularly useful in agronomy. They have however one advantage: their risk of spreading uncontrollably in fields or even to natural habitats is very low. It is very likely that such plants would be outcompeted in natural habitats where pathogens are known to fluctuate widely.

It should be noted, however, that it would be unlikely for such GM lines with inferior performance to reach the stage of commercialisation. Suitable crop lines are usually selected from a pool of several hundred or even thousands of lines. Plants with poor performance in the field, as the one's which we used here, can still be discarded at a late testing stage, i.e. after they have been moved from the controlled environment to the field.

Diversity effects

Besides the influence of the fungal infection treatments, we studied how the genetic diversity of stands influenced individual plants within these. There are examples from agronomy where increased diversity leads to reduced pathogen susceptibility and transgressive overyielding (Finckh *et al*. 2000, Mundt 2002, Wolfe 2000). If crop varieties or wild plant species are mixed with each other, it is difficult if not impossible to test if particular resistance genes or other phenotypic traits are responsible for these positive diversity effects. Transgenic plants that differ only in single genes can be useful to understand such mechanisms. Hence, we planted either monocultures or mixtures of one GM with one non-GM line or two different GM lines. We found that

several fitness-related traits and plant height were influenced by the concentration of GM plants within each plot. However, almost all of these differences could be explained by the presence of a particular line in the corresponding plots. No further benefits of mixing these GM lines with each other were detected. This result is in line with the powdery mildew results discussed above. Individual plants were not less infected with this pathogen than expected from the monoculture means. The amount of powdery mildew infection seemed to influence the overall performance of our study plants. Thus, because powdery mildew was not reduced more in plots with two GM lines than in plots with only one we would also not expect positive effects on other traits. Furthermore, high costs of resistance might have concealed such effects. Indeed, we found strong diversity effects in a sister study in which we mixed GM lines with different *Pm3* alleles (Zeller *et al*. 2012). We recommend, therefore, using more dissimilar transgenic plants for future diversity studies. Furthermore, better mixing might be necessary to obtain good diversity effects.

Conclusions

Our study demonstrates that transgenic plants may differ from their non-GM control lines in many traits and that these differences can be influenced by environmental factors (i). There were differences between the Bobwhite GM lines *Pm3b*#1 and *Pm3b*#2 as well as between the Frisal GM lines A9 *Chi* and A13 *Chi/Glu*. The latter might be explained by differences in the introduced gene construct. The lines *Pm3b*#1 and *Pm3b*#2 share, however, an identical transgene. It is most likely that different expression levels caused by positional effects were responsible for the differences between the two Bobwhite GM lines. In view of all this variation, we conclude that ecological assessments of GM plants should be done on a case-by-case basis (Andow and Zwahlen 2006).

We found that all four tested GM lines suffered from costs of resistance in the absence of the pathogen (ii). Interestingly, even transgenic lines without further increased pathogen resistance compared to already resistant control lines (variety Frisal) showed such negative effects. However, in the presence of the pathogen, three of the four tested GM lines did not differ in their performance from the non-GM control lines. In this case positive effects of the pathogen resistance probably compensated for the negative effects of costs of resistance.

Finally, the diversity of the plant communities influenced pathogen levels and plant performance (iii). However, no synergistic effects were detected. We conclude that the balance between costs and benefits of increased pathogen resistance and therefore the performance of GM plants depends mainly on environmental factors. It is conceivable that transgenic plants with high costs of resistance can outperform conventional lines only in areas with constantly high pathogen pressure. Pathogen populations are known to vary from year to year depending mostly on weather conditions and other factors. Hence, in years of low pathogen pressure, non-resistant plants should have an advantage over resistant plants. One could therefore recommend cultivating both resistant and non-resistant plants in places with variable pathogen populations.

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Figures

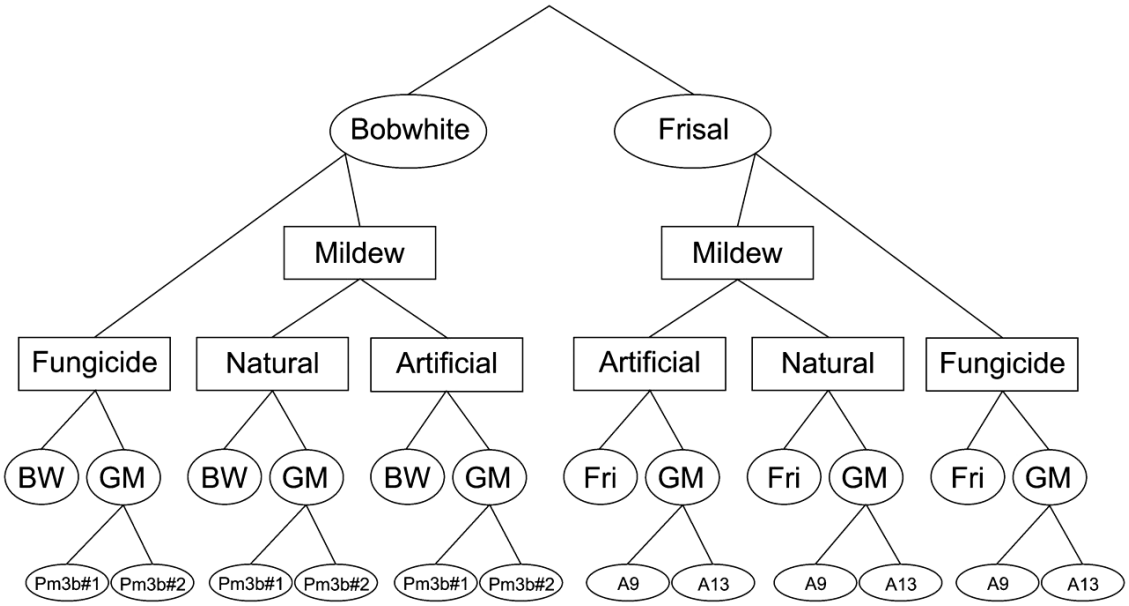


Figure 1. Hierarchical line/treatment model used in the analysis. Circles indicate varieties or lines whereas rectangles represent treatments.

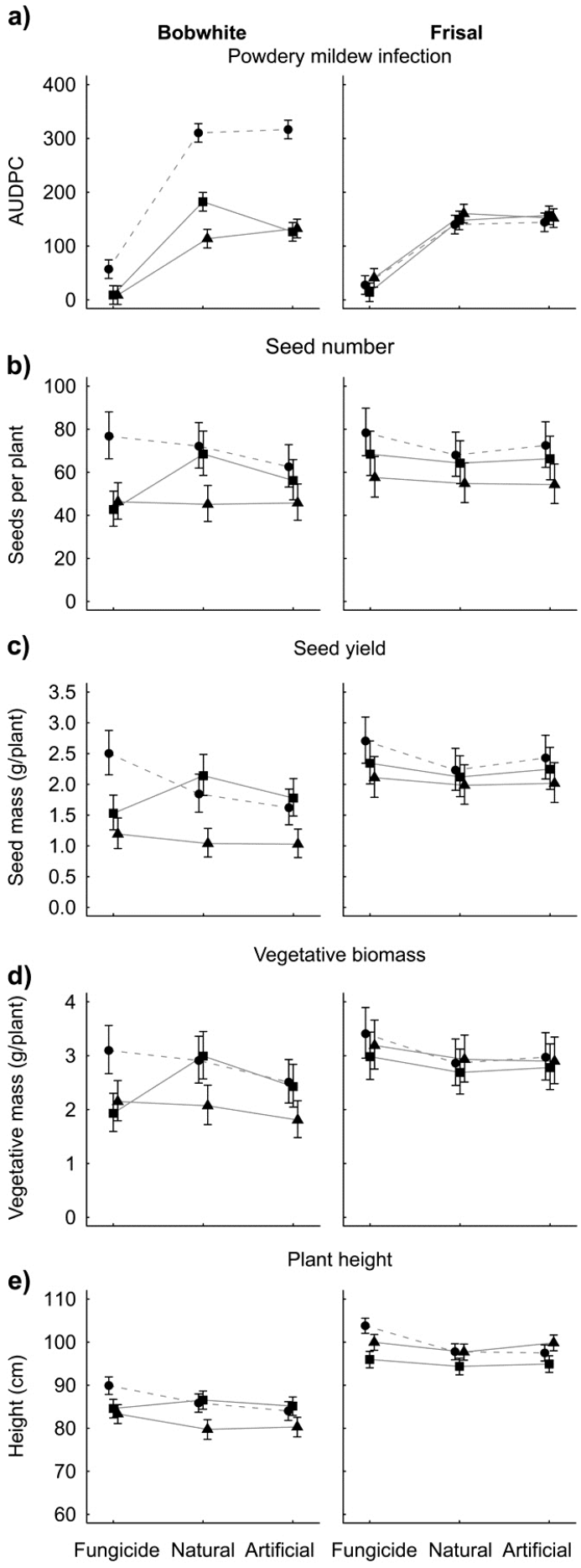
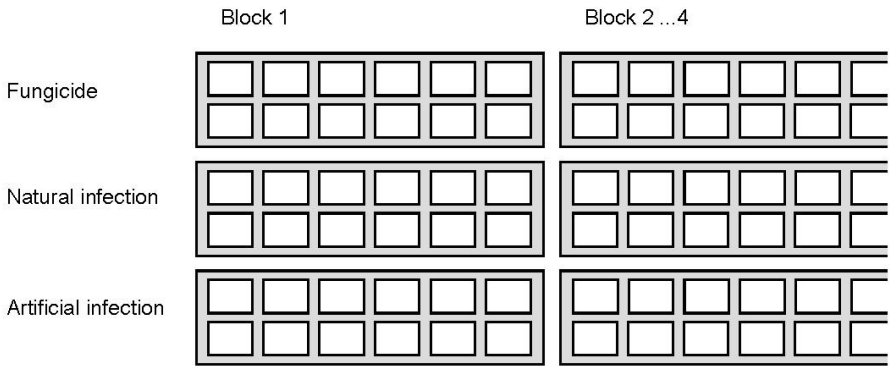

a)
Bobwhite
Frisal
Powdery mildew infection
AUDPC
400
300
200
100
0
b)
Seed number
Seeds per plant
100
80
60
40
20
0
c)
Seed yield
Seed mass (g/plant)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
d)
Vegetative biomass
Vegetative mass (g/plant)
4
3
2
1
0
e)
Plant height
Height (cm)
110
100
90
80
70
60
Fungicide
Natural
Artificial
Fungicide
Natural
Artificial

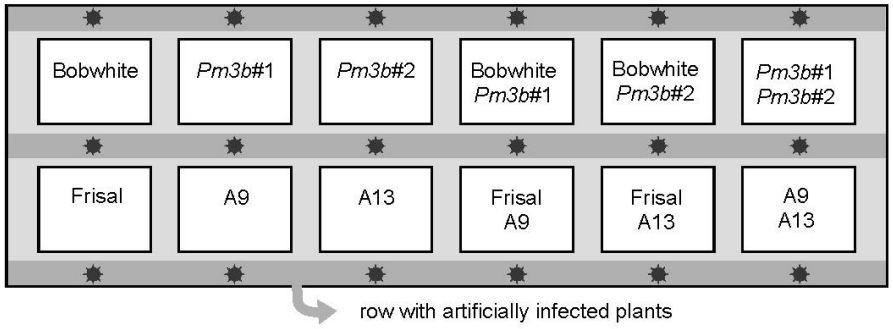
Figure 2. Effects of fungicide and natural and artificial powdery mildew infection on performance of GM and non-GM wheat. The left column shows the non-transgenic variety Bobwhite (dashed line, round symbols) and two transgenic lines *Pm3b*#1 (solid lines, square symbols) and *Pm3b#2* (solid lines, triangular symbols). The right column shows the non-transgenic variety Frisal (dashed line, round symbols) and two transgenic lines A9 *Chi* (solid lines, square symbols) and A13 *Chi/Glu* (solid lines, triangular symbols). A–E present the level of powdery mildew infection, seed number, seed yield, vegetative mass and plant height. Light grey lines were drawn to make transgene × fungal infection treatment interactions visible; error bars represent ± 1 standard error (back-transformed, see Material and Methods) and are sometimes hidden behind the symbols.

Supplemental Material to Chapter 5

A. Block and treatment structure



B. Plots in treatment (example artificial infection)



C. Seed islands in plot (example Bobwhite / *Pm3b*#1 mixture)

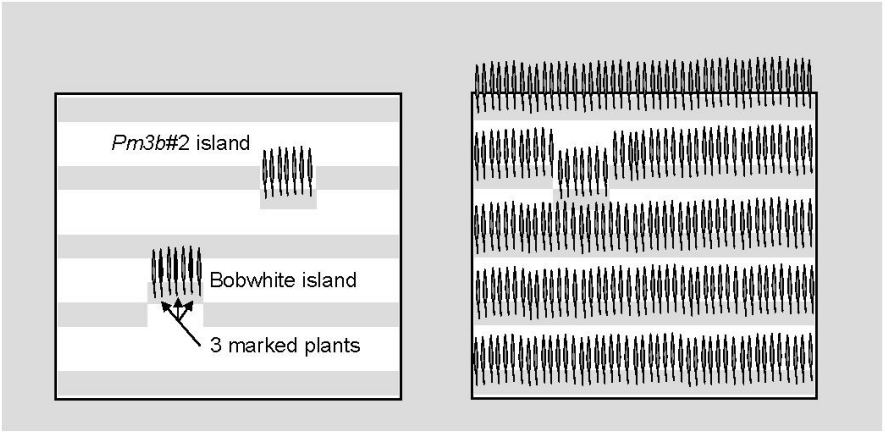


Figure S1. Experimental design of the field trial.

Table S1. Hierarchical model: summary of REML tables of AUDPC, seed number, seed yield, vegetative mass and plant height. GM richness and GM concentration were alternated. Random terms are not included in the table since their variance components are estimated directly in the REML analyses. The percentage of Wald statistic thus was calculated only for the total of the fixed effects. The total is smaller than 100% because complex interactions were omitted in this table.

	Source of variation		AUDPC		Seed number		Seed yield		Vegetative mass		Plant height	
		df	Wald %	Chi pr.	Wald %	Chi pr.	Wald %	Chi pr.	Wald %	Chi pr.	Wald %	Chi pr.
	Bobwhite vs. Frisal	1	3.4	<0.001***	8.8	0.026*	32.8	<0.001***	29.8	<0.001***	80.3	<0.001***
Variety Bobwhite (BW)	Fungicide vs. Mildew	1	10.6	<0.001***	1.6	0.349	0.4	0.527	1.1	0.494	0.7	0.095
	BW/GM in Fungicide	1	2.5	<0.001***	32	<0.001***	22.2	<0.001***	24.9	<0.001***	2.2	0.002**
	Pm3b#1/2 in Fungicide	1	0	0.896	0.3	0.672	3	0.082	0.8	0.562	0.1	0.423
	Natural vs. Artifical	1	0.1	0.577	1.8	0.818	1	0.316	4.8	0.163	0	0.69
	BW/GM in Natural	1	27.8	<0.001***	10.3	0.016*	2.6	0.108	5.7	0.122	0.7	0.086
	Pm3b#1/2 in Natural	1	2.2	<0.001***	9.2	0.022*	16.7	<0.001***	7.4	0.077	1.8	0.005**
	BW/GM in Artificial	1	41	<0.001***	3.4	0.165	0.9	0.347	3.3	0.242	0	0.767
	Pm3b#1/2 in Artificial	1	0.4	0.118	4.1	0.128	10.4	<0.001***	10.9	0.033*	1.2	0.025*
Variety Frisal	Fungicide vs. Mildew	1	10.5	<0.001***	1.5	0.369	1.3	0.256	4.4	0.187	2.2	0.003**
	Frisal/GM in Fungicide	1	0	0.678	8.7	0.026*	4	0.043*	3.7	0.216	4	<0.001***
	A9/A13 in Fungicide	1	0.4	0.127	3.2	0.176	0.6	0.425	0.8	0.561	1.8	0.005**
	Natural vs. Artifical	1	0	0.835	0.2	0.731	0.3	0.573	0.2	0.788	0.1	0.609
	Frisal/GM in Natural	1	0.5	0.091	1.7	0.32	0.2	0.625	0	0.992	0.3	0.264
	A9/A13 in Natural	1	0.1	0.49	3.7	0.146	0.5	0.47	0.6	0.624	0.7	0.077
	Frisal/GM in Artificial	1	0.1	0.397	5.4	0.08	1.7	0.183	0.6	0.602	0	0.872
	A9/A13 in Artificial	1	0.1	0.435	3.4	0.164	0.5	0.459	0.3	0.722	3.1	<0.001***
Diversity	GM richness	1	0	0.835	0.2	0.748	0.2	0.641	0.4	0.687	0	0.853
	GM concentration	1	1.6	0.208	0.6	0.557	0.5	0.466	0.3	0.739	0.8	0.073
	GM concentration	1	0.1	0.463	0.1	0.849	0	0.849	0	0.959	0.3	0.253
	GM richness	1	0.2	0.296	0.7	0.521	0.7	0.399	0.6	0.603	0.5	0.163

Table S2. Factorial model: summary of REML tables of AUDPC, seed number, seed yield, vegetative mass and plant height. GM richness and GM concentration were alternated. Random terms are not included in the table since their variance components are estimated directly in the REML analyses. The percentage of Wald statistic thus was calculated only for the total of the fixed effects. The total is smaller than 100% because complex interactions were omitted in this table.

	Source of variation		AUDPC		Seed number		Seed yield		Vegetative mass		Plant height	
		df	Wald %	Chi pr.	Wald %	Chi pr.	Wald %	Chi pr.	Wald %	Chi pr.	Wald %	Chi pr.
	Bobwhite vs. Frisal	1	3.4	<0.001***	8.8	0.026*	32.8	<0.001***	29.8	<0.001***	80.3	<0.001***
Variety Bobwhite (BW)	Fungicide/Mildew	1	10.6	<0.001***	1.6	0.349	0.4	0.527	1.1	0.494	0.7	0.095
	Natural/Artificial infection	1	0.1	0.577	1.8	0.318	1	0.316	4.8	0.163	0	0.69
	BW/GM	1	58.6	<0.001***	38.2	<0.001***	17.5	<0.001***	28.1	<0.001***	2	0.003**
	Pm3b#1/2	1	0.3	0.215	6.7	0.051	27.1	<0.001***	8.6	0.058	2.6	<0.001***
	Fung/Mild×BW/GM	1	12	<0.001***	6.5	0.054	7.9	0.005**	5.7	0.121	0.7	0.088
	Fung/Mild×*Pm3b*#1/2	1	0.1	0.474	6.4	0.056	2.5	0.109	10.2	0.038*	0.5	0.16
	Nat/Art×BW/GM	1	0.6	0.053	0.9	0.465	0.2	0.629	0.2	0.779	0.2	0.314
	Nat/Art×*Pm3b*#1/2	1	2.3	<0.001***	0.5	0.599	0.3	0.565	0.2	0.77	0	0.695
Variety Frisal	Fungicide/Mildew	1	10.5	<0.001***	1.5	0.369	1.3	0.256	4.4	0.187	2.2	0.003**
	Natural/Artificial infection	1	0	0.835	0.2	0.731	0.3	0.573	0.2	0.788	0.1	0.609
	Frisal/GM	1	0.5	0.088	14.5	0.002**	4.9	0.026*	2.5	0.307	2.3	0.002**
	A9/A13	1	0.1	0.409	10.3	0.015*	1.7	0.191	1.6	0.41	5.2	<0.001***
	Fung/Mild×Frisal/GM	1	0.1	0.485	0.8	0.492	0.8	0.367	1.5	0.428	1.9	0.004**
	Fung/Mild×A9/A13	1	0.3	0.201	0	0.953	0	0.958	0	0.898	0	0.961
	Nat/Art×Frisal/GM	1	0.1	0.549	0.5	0.598	0.3	0.554	0.3	0.717	0.1	0.499
	Nat/Art×A9/A13	1	0.2	0.298	0	0.963	0	0.992	0	0.924	0.4	0.176
Diversity	GM richness	1	0	0.835	0.2	0.748	0.2	0.641	0.4	0.687	0	0.853
	GM concentration	1	0.3	0.208	0.6	0.557	0.5	0.466	0.3	0.739	0.8	0.073
	GM concentration	1	0.1	0.463	0.1	0.849	0	0.849	0	0.959	0.3	0.253
	GM richness	1	0.2	0.296	0.7	0.521	0.7	0.399	0.6	0.603	0.4	0.163

CHAPTER 6

Gene Flow in Genetically Modified Wheat

Silvan Rieben, Olena Kalinina, Bernhard Schmid, Simon L. Zeller. 2011. PLoS ONE 6: e29730

Spike morphology in GM line *Pm3b*#2 and variety Bobwhite

Abstract

Understanding gene flow in genetically modified (GM) crops is critical to answering questions regarding risk-assessment and the coexistence of GM and non-GM crops. In two field experiments, we tested whether rates of cross-pollination differed between GM and non-GM lines of the predominantly self-pollinating wheat *Triticum aestivum*. In the first experiment, outcrossing was studied within the field by planting "phytometers" of one line into stands of another line. In the second experiment, outcrossing was studied over distances of 0.5–2.5 m from a central patch of pollen donors to adjacent patches of pollen recipients. Cross-pollination and outcrossing was detected when offspring of a pollen recipient without a particular transgene contained this transgene in heterozygous condition. The GM lines had been produced from the varieties Bobwhite or Frisal and contained *Pm3b* or *chitinase*/*glucanase* transgenes, respectively, in homozygous condition. These transgenes increase plant resistance against pathogenic fungi. Although the overall outcrossing rate in the first experiment was only 3.4%, Bobwhite GM lines containing the *Pm3b* transgene were six times more likely than non-GM control lines to produce outcrossed offspring. There was additional variation in outcrossing rate among the four GM-lines, presumably due to the different transgene insertion events. Among the pollen donors, the Frisal GM line expressing a *chitinase* transgene caused more outcrossing than the GM line expressing both a *chitinase* and a *glucanase* transgene. In the second experiment, outcrossing after cross-pollination declined from 0.7–0.03% over the test distances of 0.5–2.5 m. Our results suggest that pollen-mediated gene flow between GM and non-GM wheat might only be a concern if it occurs within fields, e.g. due to seed contamination. Methodologically our study demonstrates that outcrossing rates between transgenic and other lines within crops can be assessed using a phytometer approach and that gene-flow distances can be efficiently estimated with population-level PCR analyses.

Introduction

The frequent use of genetically modified (GM) plants in agriculture demands in-depth ecological risk assessment (Wolfenbarger and Phifer 2000, Cellini *et al.* 2004, Conner *et al.* 2003, Snow *et al.* 2005, Andow and Zwahlen 2006, EFSA 2010). A possible consequence of the release of GM crops can be unintended gene flow to non-GM conspecifics or to wild relatives (Jørgensen and Andersen 1994, Daniell 2002, Rieger *et al.* 2002, Mercer and Wainwright 2008, Schoenenberger *et al.* 2006, Piñeyro-Nelson *et al.* 2009). Gene flow can increase the ability of a population to respond to a changing environment due to increased genetic diversity (Gustafson *et al.* 2005). In plants, gene flow occurs not only by migrating individuals (seed dispersal) but also by migrating gametes, i.e. pollen dispersal. Gene flow via pollen dispersal can occur within and between populations and occasionally even between species (Levin and Kerster 1974, Hedrick 2004). Understanding this process is critical to ensuring the coexistence without gene exchange of GM and non-GM crops (Weber *et al.* 2007, Pla *et al.* 2006). In particular, the data about pollen-mediated gene flow are essential to establish appropriate isolation distances between the two (Waines and Hegde 2003). In practice, isolation distances should be large enough to achieve the European Union (EU) GM-adventitious-presence-labeling threshold for food and feed, which allows a maximum contamination of 0.9% GM material in non-GM produce (Beckie and Hall 2008).

Previous studies about gene flow in conventional wheat, a predominantly self-pollinating species (De Vries 1971), have found cross-pollination rates of 1–2% for plants in close proximity (De Vries 1974, Griffin 1987, Martin 1990, Gustafson *et al.* 2005), which rapidly decreases with greater distance between pollen donor and pollen recipient (De Vries 1971, Gatford *et al.* 2006). However, Lawrie *et al.* found that cross-pollination rates, using direct spike contact inside glassine bags, could exceed 10% (Lawrie *et al.* 2006). There are several reasons why wheat has a low cross-pollination rate compared to other grain species. First, fertilization usually occurs before the florets open, which makes pollination with foreign pollen unlikely. Second, although wheat is a wind-pollinated species (Eastham and Sweet 2002), its pollen is relatively heavy and settles quickly compared to other grass species (De Vries 1971). Despite the low rates of gene flow, a maximum cross-pollination distance of 2.75 km has been reported in the literature (Matus-Cádiz *et al.* 2007).

While there are numerous studies about gene flow over certain distances, gene flow within stands of crop plants, including wheat, has rarely been analyzed. Such

studies are necessary to assess the potential dispersal of GM traits if GM plants occur as contamination within fields planted with non-GM crops, due to contaminated seed material or volunteer seedlings (Graziano *et al.* 2007). It is usually assumed that GM-wheat would behave similar to conventional varieties, but only scant evidence corroborates this standpoint (Gatford *et al.* 2006).

In the present study we used GM and non-GM lines of spring wheat *Triticum aestivum* L. with transgenes conferring resistance against fungal pathogens as a model system to assess gene flow by cross-pollination within stands and over short distances in two field experiments. To assess gene flow within the field, we planted seedlings of four independently transformed *Pm3b* and corresponding non-GM control lines as "phytometers" (Clements and Goldsmith 1924, Zeller *et al.* 2010, Kalinina *et al.* 2011) into plots with four different wheat varieties (experiment 1). The low density of phytometer relative to other plants ensured a high "cross-pollination pressure" from the latter and mimicked a situation of the presence of adventitious GM plants in a non-GM background. Outcrossing events were identified by the hybrid phenotype of plants raised from the seeds produced by phytometer plants. To assess gene flow over short distances, we grew 2.5 m strips of non-GM control lines east and west of 1 × 1 m GM wheat plots. In this second experiment we determined the cross-pollination rate by pooling offspring seeds from the control lines and testing them for the presence or absence of resistance genes using population-level molecular analyses.

The aims of the study were to (i) measure gene flow within the field from two GM and two non-GM lines planted as pollen-donor backgrounds to four different pairs of GM/non-GM sister lines planted as pollen-recipient phytometers, (ii) to measure the influence of distance between GM pollen donor and non-GM pollen recipient on the cross-pollination rates of three pairs of GM/non-GM sister lines and (iii) to analyze line-specific differences in rates of cross-pollination.

Materials and Methods

Genetically modified wheat

We used six GM lines of spring wheat either derived from the Mexican variety Bobwhite SH 98 26 or the Swiss variety Frisal. Four GM lines from the variety Bobwhite SH 98 26 were produced by biolistic transformation in different transformation events and each line carried a single copy of the transgene *Pm3b* (Zeller *et al.* 2010). *Pm3b* confers race-specific resistance to powdery mildew and was cloned

from hexaploid wheat (Yahiaoui *et al.* 2004). The transgene was cloned under the control of the maize *Zea mays* L. ubiquitin promoter (Christensen and Quail 1996) and transformants were selected on mannose-containing media using the phosphomannose isomerase (PMI) coding gene as a selectable marker (Reed *et al.* 2001). After regeneration of T_0 transformants, four independent T_1 families were selected. From each T_1 family, an offspring pair was further propagated consisting of a homozygous GM plant (GM lines *Pm3b*#1–4) and a null-segregant, i.e. a plant that inherited neither the *Pm3b* transgene nor the selectable marker (control lines Sb#1–4; Zeller *et al.* 2010).

Two GM lines of the variety Frisal were produced by biolistic transformation using the plasmid MAGUCUM, containing (1) an actin-1 promoter, barley-seed ß-1,3-glucanase (*glu*) and CaMV terminator, (2) an ubiquitin-1 promoter, barley-seed chitinase (*chi*), CaMV terminator and (3) the bar gene for selection (Bliffeld *et al.* 1999). The GM line A9 *Chi* was positively selected for chitinase expression and the line A13 *Chi/Glu* for chitinase and glucanase expression (Bieri *et al.* 2003). The pathogenesis-related proteins chitinase and glucanase are known for their broad antifungal effect and their expression should lead to an increased resistance to powdery mildew (Leah *et al.* 1991, Zhu *et al.* 1994). Because for the GM-lines of Frisal we did not have null-segregants, it is conceivable that the differences between GM and non-GM lines in Frisal were not only due to the insertion of the transgene but also to additional events that occurred during transformation, e.g. soma-clonal variation acquired during tissue culture.

For the field experiments we used seeds obtained from the fifth generation of the GM lines *Pm3b*#1–4 and their respective non-GM sister lines Sb#1–4 as controls, and seed obtained from the sixth generation of the GM lines A9 *Chi* and A13 *Chi/Glu* and its cultivar Frisal as a control. In addition we used the conventional wheat variety Casana as a further non-GM control line.

Experiment 1: gene flow within plots

The first part of experiment 1 was a field trial with GM and non-GM wheat lines running from March 2008 until August 2008 at an agricultural research station in Zurich-Reckenholz, Switzerland (Zeller *et al.* 2010). Seeds of the variety Frisal, its GM lines A9 *Chi* and A13 *Chi/Glu*, and the variety Casana, were sown into eight 1 × 1.08 m plots per line. These stands acted as pollen-donating wheat backgrounds. In each plot, 400 seeds were sown in six rows with a distance of 18 cm between rows using an

Oyjord plot drill system (Wintersteiger AG, Ried, Austria). At the same time, seedlings of the four *Pm3b* lines and the four corresponding control lines (Sb#1–4) were raised individually in the glasshouse and transplanted as "phytometers" (Kalinina *et al.* 2011) into each of the 32 field plots once they showed two or three unfolded leaves. Each of the eight lines was represented by two phytometer plants per plot, resulting in a mixing ratio of 16 transplanted phytometers per 400 sown background plants. With this planting procedure we aimed to maximize chances for pollen transfer from background to phytometer plants. Furthermore, it allowed us to detect outcrossed offspring later on because hybrids between Frisal or Casana and Bobwhite differ morphologically from the parental varieties. The flowering period of background and phytometer plants was continuously recorded. After seed maturation, all phytometer plants were individually harvested and threshed. Seeds originating from a single phytometer mother plant are called seed family in the following text. Four of the eight replicate field plots per background line received fertilizer twice during the growing season, i.e. when the plants unfolded the first leaf and when the flag leaf became visible (each time 3 g N m^{-2} were applied as "Ammonsalpeter 27.5", Lonza, Visp, Switzerland; see Zeller *et al.* 2010 for further details of field design).

The second part of experiment 1 took place from March–August in 2010. We planted offspring seeds of the eight phytometer lines from the field experiment 2008 back to the field site. Only phytometer plants that had flowered at the same time as the corresponding pollen-donating background plants and which produced at least four seeds were used. In total, 146 out of 265 seed families (4 blocks × 2 fertiliser treatments × 4 background lines × 8 phytometer lines) met these criteria. A minimum of 4 and a maximum of 16 seeds were planted from each seed family, resulting in a total of 1945 individual offspring. We sowed the seeds in patches of four per seed family into ten plots of 1 × 4 m by hand. The patches were assigned to positions and plots in a completely randomised design. The positions within a plot formed a grid of three rows with a distance of 18 cm between patches (60 seed patches per plot). The plots were arranged in a grid aligned along an x-axis leading from east to west and a y-axis leading from south to north. The plots were surrounded by additional buffer plants of variety Bobwhite to avoid edge effects on the test plants. Phosphorus and potassium fertiliser had been applied to the plots prior to the seed planting in autumn 2009 at a rate of 46 kg P_2O_5 ha^{-1} and 60 kg K_2O ha^{-1}. The amount of mineralised nitrogen, determined at the end of February 2010 in the top 100 cm of the soil was 41.7 kg N ha^{-1}. Nitrogen

fertiliser was additionally applied immediately after sowing (30 kg N ha^{-1}) and another 30 kg N ha^{-1} when the flag leaves of the plants became visible. All plots were sprayed with the herbicide cocktail Concert SX (40% Thifensulfurone, 4% Metusulfurone-methyl; Stähler Suisse AG, Zofingen, Switzerland) on 18 May.

We determined the cross-pollination rate by dividing the number of mature offspring hybrids through the total number of mature plants per phytometer. Hybrids produced by cross-pollination of Bobwhite by Frisal or Casana differed visibly in their morphology from offspring produced by self-pollination or cross-pollination with other Bobwhite plants. They were taller and had a reduced awn length than the parental varieties and suffered from slight hybrid necrosis, which can occur when unrelated wheat varieties are crossed (Hermsen 1963). It should be noted that our cross-pollination rate is equivalent to outcrossing rate, that is, we only counted successful pollination with subsequent seed set and offspring growth as pollination event.

To check the reliability of the morphological assessment of hybrid status, all plant classified as hybrids and 65 randomly chosen plants not classified as hybrids (= putatively selfed offspring) were tested for the presence or absence of the transgenes *Pm3b*, *chi* and *chi/glu* using Polymerase Chain Reaction (PCR) analysis. DNA was isolated from 200–300 mg of fresh leaf tissue by adapting the method of Stein *et al.* (Stein *et al.* 2001). For the amplification of the *Pm3b* gene, we chose primer sequences fitting the ubiquitin promoter (5'-ATCTCTGTCGCTGCCTCTGG-3' and 5'-TGTGC-GCTCCGAACAACACG-3'; Sigma-Aldrich GmbH, Buchs, Switzerland). The *Chi/Glu* transgenes were detected by amplification of parts of the *bar* gene in the MAGUCM plasmid ('5-TCAACCACTACATCGAGACA-3' and '5-AGTCCAGCTGCCAGA-AAC-3'; Sigma-Aldrich GmbH, Buchs, Switzerland). The amplified DNA was separated and visualized performing gel electrophoresis. In total, 97.5% of the hybrids and the putatively selfed offspring were identified correctly, based on the presence/absence tests of *Pm3b*, *chi*, and c*hi/glu* transgenes (data not shown). We conclude therefore that the method of hybrid detection by visual phenotyping was appropriate.

Experiment 2: Short-distance gene flow between adjacent subplots

The second field experiment took place at the same agricultural research station as experiment 1 and lasted from March until August 2009. Three GM lines *Pm3b*#1, *Pm3b*#2 and A9 *Chi* and their corresponding non-GM lines Sb#1, Sb#2 and Frisal were

grown in three separate 7 × 1 m cross-pollination plots (Figure S1 in Supplemental Material). Each plot consisted of one subplot (1 × 1 m) in the centre with GM plants as pollen donors and four subplots (0.5 × 1 m) on two opposing sides with the corresponding non-GM plants as pollen recipients. The opposing sides were in eastern or western direction of the pollen source because the prevailing winds at the field site blow from the west (see Figure S1 in Supplemental Material). The distances between central subplot and side subplots were 0–0.5, 0.5–1, 1–1.5 and 2–2.5 m (a subplot also occurred between 1.5–2 m but was not harvested). As there were four replicate blocks × eight subplots with pollen recipients (distance subplots) x three line combinations, the sample size was 32 for each tested line and 96 in total. The distance subplots were sown with an Oyjord plot drill system (Wintersteiger AG, Switzerland) and the central plots with the GM pollen source was sown by hand. Seeding density was 400 seeds m^{-2} and there were six rows spaced 18 cm apart. The cross-pollination plots were at least 2 m apart from each other and the intervals were filled with tall-growing triticale plants acting as a pollen barrier to minimize cross-pollination between plots. Flowering periods of pollen donor and receptor subplots were similar in order to allow cross-pollination. Nitrogen fertilizer was applied one day before sowing (40 kg N ha^{-1}) on 25 March and again when the plants had their first leaf unfolded (30 kg N ha^{-1}). Phosphorus and potassium fertiliser were applied twice at a rate of 46 kg P_2O_5 ha^{-1} and 60 kg K_2O ha^{-1} when the plants unfolded the first leaf and when the flag leaf became visible. The plots were sprayed with the herbicide cocktail Concert SX (40% Thifensulfurone, 4% Metusulfurone-methyl; Stähler Suisse AG) and Starane super (120 g l^{-1} Bromoxynil, 120 g l^{-1} Ioxynil, 100 g l^{-1} Fluroxypyr-metilheptil-ester; Omya Agro AG, Safenwil, Switzerland) in the beginning of May. The plots were treated twice with the insecticide Karate Zeon (100g l^{-1} Lambda-Cyhalothrin; Syngenta Agro AG, Dielsdorf, Switzerland) against the wheat stem fly (*Chlorops pumilionis* Bjerk.) in the beginning of May and 2 weeks later.

To measure the cross-pollination (and outcrossing) rate in each distance subplot we used a population-level PCR analysis that detected the transgenes *Pm3b* and *chi* in batches of seeds. A single-seed approach was not feasible due to the low expected cross-pollination rates. The optimal size of seed batches was determined in a pilot study with flour from seed batches of defined numbers of GM and non-GM seeds. PCR amplification of DNA extracted from flour of the different seed batches showed that a single GM seed could be detected reliably in 1:10, 1:50, 1:200 and 1:500 mixtures of

GM:non-GM seeds. Potential outcrosses would be heterozygous and would therefore contain only 50% of the DNA of a homozygous GM seed. Taking this into account, we opted for seed batches of 100 seeds in our experiment 2 (Figure S2 in Supplemental Material).

For the analysis of the cross-pollination rate, we collected 5 batches of 100 seeds per distance subplot and produced flour from each batch (TissueLyser, Qiagen Instruments AG, Hilden, Germany). To avoid DNA contamination between batches, the jars used for the milling were sprayed with DNA-ExitusPlusTM (AppliChem GmbH, Darmstadt, Germany) and incubated at 60 °C for 10 min to increase the degradation rate of DNA (Esser *et al.* 2006).

DNA was extracted from 20 mg flour per sample adapting the method of Kang *et al.* (Kang *et al.* 1998). To test the DNA extracts for transgene-presence we used the same PCR protocol as described above. If a sample tested positive, DNA extraction and PCR were repeated. Positive samples were therefore based on at least two independent DNA extractions and PCR reactions (Figure S3 in Supplemental Material).

Data analysis

The influence of background and phytometer lines on cross-pollination within the plot, measured as the probability of an individual offspring plant to be a hybrid rather than a putatively selfed offspring (experiment 1) was analyzed using generalized linear models (GLMs) with logit link function and binomial error distribution (McCullagh and Nelder 1989). To account for potential overdispersion, experimental factors were tested with approximate F-tests derived from analysis of deviance tables (Crawley 2007; see Table S1 in Supplemental Material). Experimental factors were block, fertilizer application, phytometer line with the four contrasts *Pm3b* vs. control, *Pm3b*#2 vs. other three *Pm3b* lines, variation among these three *Pm3b* lines and variation among the four control lines, background line with the three contrasts Frisal vs. Casana, Frisal GM vs. Frisal control and Frisal A9 vs. Frisal A13, interactions among these terms and phytometer individual (seed family; "Residual" in Table S1 in Supplemental Material). Plants that did not germinate or died due to pest infestation were excluded from further analysis.

Data from experiment 2, the short-distance gene-flow experiment, were analyzed using GLMs with logit-link function and binomial error distribution (Table S2 in Supplemental Material). The dependent variable was the probability to find a transgene in a batch of 100 seeds. In one model, the experimental factor distance was

decomposed into a contrast log (distance) and residual variation between distance classes because cross-pollination rates are likely to decrease logarithmically with increasing distance to the pollen source (Albrecht *et al.* 2009). To investigate differences between very short (0–0.5 m) and short-distance (0.5–2.5 m) gene flow, we split the dataset and analyzed both subsets separately. The highest possible estimate of cross-pollination rate was calculated by dividing the observed amount of positive batches by the total amount of batches. This makes the highly unlikely assumption that in all positive batches all 100 seeds result from cross-pollination. The lowest possible estimate of cross-pollination rate was calculated by dividing the positive samples by the total amount of samples multiplied by 100. This makes the assumption that in all positive batches only 1 seed out of 100 is the result of cross-pollination. Following the maximum likelihood estimation for binomial data, we calculated the values most likely to have produced the observed results (Fisher 1922). The estimate for the probability is: $p = 1 - ((n–z)/n)^{(1/J)}$, with n being the total amount of batches, while z represents the positive batches and J the number of seeds per batch, i.e. 100. All statistical analyses were performed with the statistical software R 2.9.2 (R Development Core Team 2010). The critical significance level was 0.05 for all analyses.

Results

Experiment 1: gene flow within plots

40 out of 1192 mature plants could be identified visually as hybrids indicating that 3.36% of all planted seeds had received pollen from foreign wheat varieties (background). Overall, 14.4% of all mother plants produced at least one hybrid seed and 19.6% of all seeds of such plants were identified as hybrids. 21 out of 40 hybrids were crosses between two GM lines, leading to natural but heterozygous pyramiding of *Pm3b* and *chi* or *chi*/*glu* transgenes.

The identity of the mother line, i.e. phytometer plants, significantly influenced the hybridization rate (Table 1 and Table S1 in Supplemental Material): 7.25% of all *Pm3b* seeds were hybridized, which is 6.2 times as many as for the corresponding non-GM control lines ($P<0.001$ when tested against seed family as a residual in Table S1). There was also significant variation among the four GM lines which could be explained by a contrast between line *Pm3b*#2, which had fewer hybrids, and the other *Pm3b* lines ($P = 0.018$). This difference between lines within the group of GM lines was, however, much smaller than the difference between GM and control lines, which can be seen by

comparing the deviances in Table S1 (2.3% vs. 16.1% of total deviance). There was no significant variation among the four non-GM control lines (P = 0.4), indicating that the events during the transformation and tissue culture process did not cause additional variation among lines.

The identity of the father line, i.e. background plants, also significantly influenced the hybridization rate (Table 1 and Table S1 in Supplemental Material). Among the Frisal fathers, A9 *Chi* pollinated more plants than did A13 *Chi*/*Glu* (P<0.001 for difference A9/A13). Hybrids with Casana fathers had shorter awns than hybrids with Frisal fathers (P=0.013 for difference Casana father/Frisal father). Within fathers of the variety Frisal, plants pollinated by Frisal GM fathers (A9 *Chi*, A13 *Chi*/*Glu*) had shorter awns than when pollinated by Frisal control fathers (P=0.02 for difference Frisal A9 and A13 father/Frisal control father).

Finally, there were some significant interactions between mother and father lines (Table S1 in Supplemental Material). The Frisal control fathers pollinated more control mothers than did the Frisal GM fathers, which in turn pollinated preferably GM (=*Pm3b*) mothers (P=0.03 for interaction *Pm3b* vs. control × Frisal GM vs. Frisal control).

Experiment 2: Short-distance gene flow between adjacent subplots

Upper and lower boundaries of the estimated cross-pollination rates are shown in Figure 1A. The upper boundary shows cross-pollination rates assuming that all seeds of a 100-seed sample were genetically modified, if a single seed was tested positive, whereas the lower boundary assumes that only 1 seed in a 100-seed sample was positive. Using the log(distance) model, we found higher cross-pollination probabilities in the west than in the east (P=0.048 for difference west/east; Table S2 in Supplemental Material). Furthermore, Frisal A9 *Chi* plants were more likely to outcross than Bobwhite plants (P=0.02 for difference Bobwhite/Frisal A9 *Chi*). We found no significant differences between the two *Pm3b* lines if we combined the data of all distances. However, if we analyzed the subplots closest to the pollen source (0–0.5 m) separately, *Pm3b*#1 was more likely to outcross than *Pm3b*#2 (P=0.05 for difference *Pm3b*#1/*Pm3b*#2). Neither varieties, lines nor wind directions differed significantly in subplots further away from the pollen source (0.5–2.5m).

The actual cross-pollination rates lie between the upper and the lower boundary estimates. We calculated the most likely cross-pollination rate for each distance subplot

using a maximum likelihood method (Figure 1B). We found that the estimated overall cross-pollination rate was 0.8% in the west and 0.5% in the east if measured at a distance of 0–0.5 m from the pollen source. Cross-pollination rates decreased more or less linearly with logarithmically increasing distance to the pollen source. Nevertheless, our methods were accurate enough to detect cross-pollination events in 2.5 m distance to the pollen source. The detected rates of 0.05% in the west and 0.02% in the east would be low enough to meet the seed-purity levels set by the European Union (Beckie and Hall 2008).

Discussion

Increased gene flow of Pm3b wheat lines within the field

Large differences among wheat cultivars concerning pollen-mediated gene flow have been reported before and were often attributed to dissimilarities in male fertility and morphological traits (Waines and Hegde 2003, Lawrie *at al.* 2006, Matus-Cádiz *et al.* 2007). However, we found no other reports showing a higher rate of pollen-mediated gene flow to GM plants than to non-GM plants. Because there were additional differences among the four GM-lines of Bobwhite in our experiment 1, it is conceivable that depending on the insertion event, the particular transgene increased the outcrossing rate to a greater or lesser degree. This would be consistent with phenotypic differences among the four GM lines (Zeller *et al.* 2010, Brunner e*t al.* 2011): lines *Pm3b*#2 and #4 had, measured on other plants but in the same field trial, strongly increased levels of ergot infection, suggesting that their stigmata were exposed for a prolonged period of time which would also have increased their chances to receive foreign pollen (Zeller *et al.* 2010). The prolonged exposition of stigmata might in turn have been related to reduced male fertility of the corresponding GM-lines (Waines and Hegde 2003). However, there remains an inconsistency, because line *Pm3b*#2, which had high ergot infection and presumed reduced male fertility, actually had the lowest maternal hybridization rate compared with the other three *Pm3b* lines.

Besides the capacity to receive foreign pollen, the ability to pollinate other plants seems to be important to gene flow as indicated by the differences in pollination rates between father plants from different lines. In this case, however, the difference between Frisal non-GM and GM lines was not significant, whereas the difference between the two GM lines was highly significant. In contrast to the Bobwhite GM lines, we had no null-segregants for the Frisal GM lines. Therefore, it is more difficult

to interpret the differences among the pollen donor than among the pollen recipient lines. Finally, the significant interactions between donor and recipient lines in our experiment 1, with a higher crossing success for non-GM × non-GM and GM × GM combinations than for other combinations, hint at the complexity of genetic combining ability between specific lines (Schmid and Dolt 1994), which demonstrates the importance to test the crossing behavior of GM lines on a case-by-case basis.

We found that on average 3.36% of all tested seeds had resulted from hybridization with neighboring plants. However, this cross-pollination rate varied among the eight wheat lines tested from 0.5–8.5%. These rate measurements are critical to answer questions concerning the EU 0.9% threshold for GM seeds in the harvest (Graziano *et al.* 2007). A study with maize *Zea mays* L using a color marker showed an increased percentage of marked seeds at harvest compared to sowing (Dietiker *et al.* 2011). The contamination percentage at sowing was 1% and on average 2.8 times as high at harvest. The authors therefore concluded that contamination at sowing should be as low as 0.2–0.5% to guarantee the EU 0.9% threshold at harvest. In other words, in the case of maize a seed purity of 0.9% at sowing would not be sufficient to ensure the threshold. However, in the mainly selfing crop wheat, the increase in percentage GM seeds from sowing to harvest would be much smaller even under worst-case scenarios: assuming a cross-pollination rate of 8.5% (the maximum found above) and an initial GM proportion of 0.9%, the proportion at harvest would rise to 1.084% (seeds which are homo- or heterozygous for the transgene). As a caveat we must mention that our phytometer plants occurred at a higher frequency in their plots than would be the expected for adventitious GM plants in a non-GM crop.

Gene flow in wheat: short and random

The short-distance gene flow estimated in our experiment 2 for wheat matches the results of prior observations in which the average cross-pollination rate was about 1% in close proximity and decreased rapidly with distance from the pollen source (Gustafson *et al.* 2005). When planning our experiment, we expected to find stronger cross-pollination toward the east than toward the west, due to prevailing winds at the study site. As expected, winds mostly blew from west or northwest during flowering (data not shown). Surprisingly, however, we estimated higher cross-pollination rates in the western subplots. Data from a nearby weather station showed that there were a few hours of easterly or north-easterly winds while 23% of the mother plants were

flowering. It might be that cross-pollination occurred mainly during these hours, which then led to a higher cross-pollination in the western subplots. Gene flow also occurred mostly in the opposite direction of prevailing winds in a study by Gatford *et al.* (Gatford *et al.* 2006). We conclude, therefore, that not only prevailing winds are important for cross-pollination, but the winds at the exact time of flowering. Hence, as the time of flowering in wheat is usually short (De Vries 1971), cross-pollination can occur in all directions. This should be considered when planning cross-pollination experiments and determining isolation distances.

We could detect significant differences in gene flow between the varieties Bobwhite and Frisal over a distance of < 0.5 m. When comparing the varieties from the subplots which were at least 0.5 m away from the pollen source, no significant differences could be detected anymore. Consistent with the results from experiment 1, *Pm3b*#1 outcrossed significantly more than *Pm3b*#2 up to a distance of 0.5 m from the pollen source. We conclude therefore that the differences between varieties and lines are mainly present over short distances between pollen donor and recipient.

As a methodological corollary, our results from experiment 2 show that pooling can be an appropriate method to gain information on an entire population. Taking population samples of 100 pooled seeds turned out to be the optimal size to estimate rates of cross-pollination over short distances using a maximum-likelihood method. Pooled measures over larger distances or individual measures even over the shortest distance would have led to (too) many negative counts. Choosing the right distance allows not only determination of presence or absence of gene flow, but also an estimation of the quantity of transferred pollen is possible based on probability calculations.

Conclusions

Our results show that GM lines of wheat can differ in their outcrossing behavior from non-GM control lines. We found that Bobwhite mother plants with a *Pm3b* transgene were more likely to hybridize with other wheat varieties than were non-GM Bobwhite mother plants. This likelihood even varied among the different GM lines. One potential reason for this could have been a more or less prolonged flowering time and stigma exposition among GM lines due to more or less reduced male fertility (Waines and Hegde 2003). We also found that Frisal father plants with a *chi* transgene produced more offspring than Frisal father plants with both *chi* and *glu* transgenes, again

demonstrating different outcrossing behavior even among different GM lines. Finally, we could demonstrate that hybrids with two or even three transgenes can occur if different GM plants are planted in close proximity. Such plants could further complicate environmental risk assessments.

Because cross-pollination rates varied strongly between GM and non-GM lines and also among GM lines it may be difficult to develop universal models for pollen-mediated gene flow in wheat. Our results suggest that a case-by-case approach will be required instead (Andow and Zwahlen 2006). The gene-flow rates which we measured in our experiment 2 indicate that gene flow in wheat mainly occurs over short distances. However, within the field, 14.4% of all maternal plants received pollen from neighboring plants and 3.4% of all offspring seeds were sired by neighboring plants. Each homozygous GM plant is likely to outcross with several neighbors which will result in plants heterozygous for the transgene. The proportion of GM plants within a population is therefore likely to increase. If we take a cross-pollination rate of 3.4% and assume an initial GM contamination of 0.9%, 0.931% of all offspring seeds would contain at least one copy of the transgene. If all plants would have been cross-pollinated, this rate would increase to 1.79% in one generation. We conclude that the determination of cross-pollination rates within the field might be more important than cross-pollination over a distance in order to define appropriate threshold limits necessary to allow coexistence of GM and conventional farming systems.

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Tables

Table 1. Cross-pollination rates (mean ± 1 standard error) of the eight pollen recipient lines (Bobwhite phytometer plants) and the four pollen-donor lines (background plants). Non-GM recipient control lines (Sb#1–4) had significantly lower cross-pollination rates than GM recipient lines (*Pm3b*#1–4). The GM line *Pm3b*#2 with highest transgene expression and lowest fertility had significantly lower cross-pollination rates than the other recipient GM lines. Frisal and Casana are non-GM wheat varieties; A9 *Chi* and A13 *Chi/Glu* are GM lines based on the variety Frisal. The GM line A9 pollinated significantly more phytometer plants than did GM line A13. Cross-pollination is defined as number of seeds derived from cross-pollination divided by number of all seeds x 100.

Non-GM recipient lines		GM recipient lines		Donor lines	
Sb#1	1.43 ± 0.08	*Pm3b*#1	6.56 ± 0.83	Frisal	2.67 ± 0.10
Sb#2	0.75 ± 0.03	*Pm3b*#2	0.76 ± 0.26	Casana	3.39 ± 0.50
Sb#3	1.90 ± 0.09	*Pm3b*#3	7.24 ± 0.74	A9 *Chi*	6.16 ± 0.41
Sb#4	0.50 ± 0.02	*Pm3b*#4	8.52 ± 0.76	A13 *Chi/Glu*	0.61 ± 0.08
mean	0.55 ± 0.06		5.77 ± 0.65		3.21 ± 0.27

Figures

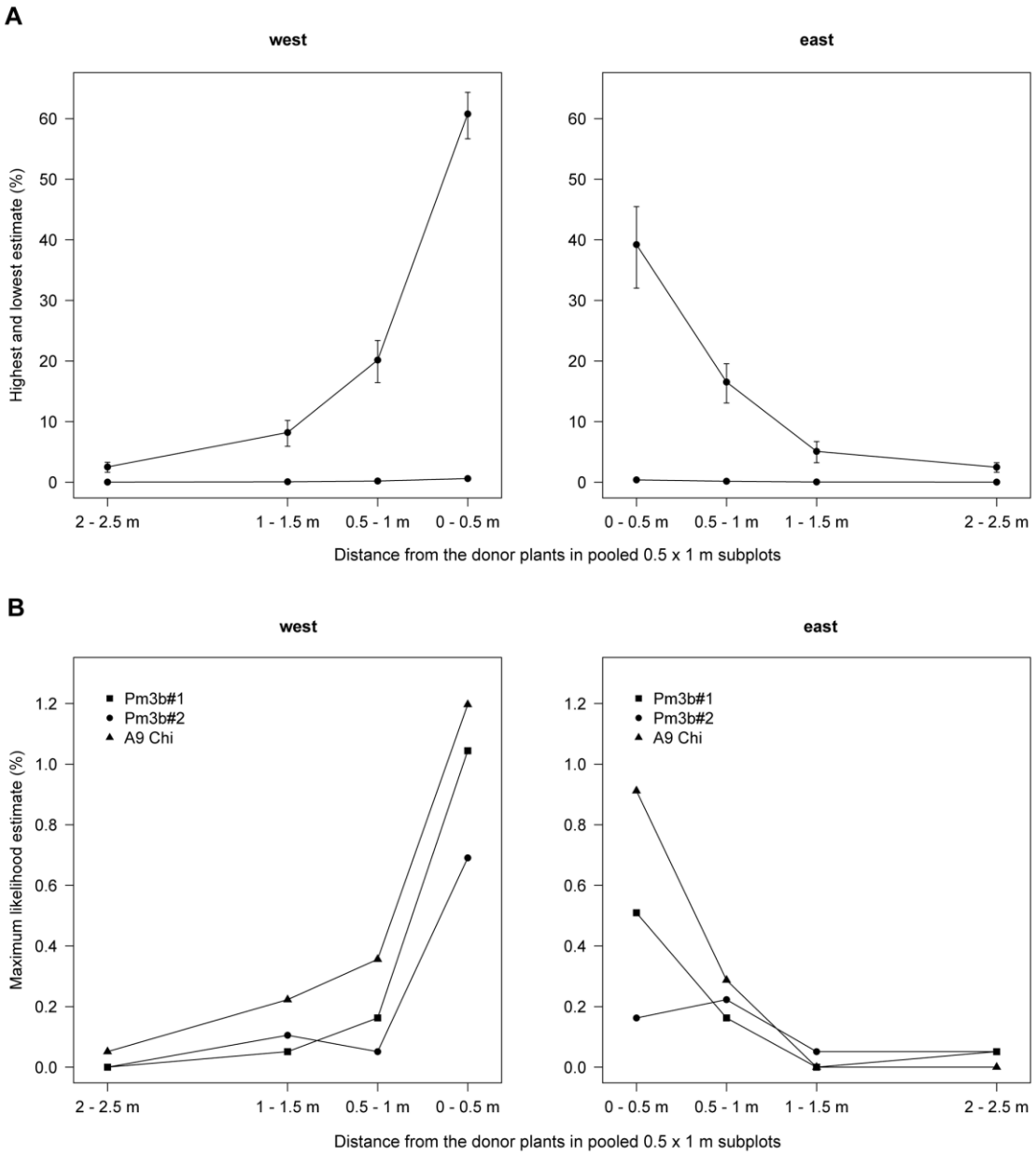

Figure 1. Cross-pollination of GM wheat over short distances and in two wind directions. A: Upper and lower boundaries of cross-pollination rate estimates (mean±1 SE, back-transformed from logit scale) for western and eastern distance subplots. Data from all lines were pooled. B: Maximum likelihood estimate of cross-pollination rate for the western and eastern subplots for the lines *Pm3b*#1, *Pm3b*#2 and A9 C*hi*. These estimates indicate cross-pollination rates between 1.2% and 0.16% in the closest and 0.05% and 0.0% in the farthest subplots.

Supplemental Material to Chapter 6

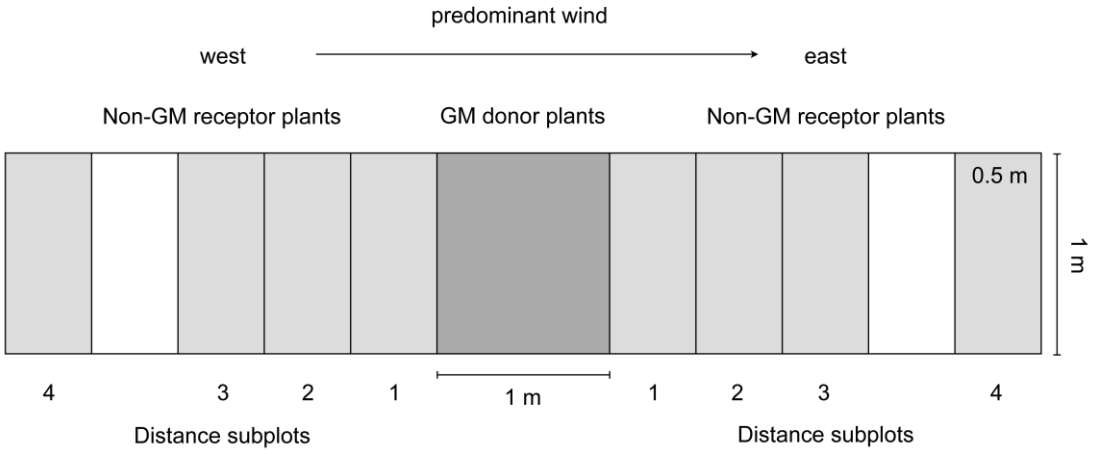

Figure S1. Schematic design of a cross-pollination plot. In the centre a 1 m^2 quadratic subplot of GM wheat was sown as a pollen source. In the eastern and western direction corresponding non-GM plants were sown as pollen recipients into distance subplots (0.5 × 1 m). The lightly shaded distance subplots were harvested after seed maturation.

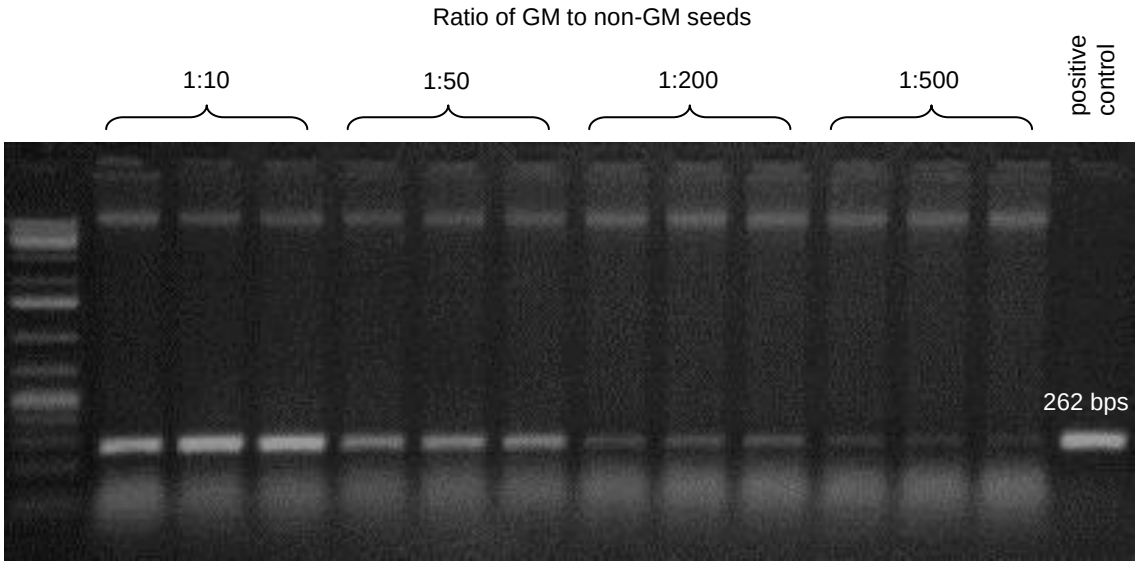

Figure S2. PCR analysis from flour of different seed mixtures containing 10%, 2%, 0.5% and 0.2% GM seeds. The positive bands show decreasing signal strength as the proportion of GM seed material decreases. Each analysis was replicated three times.

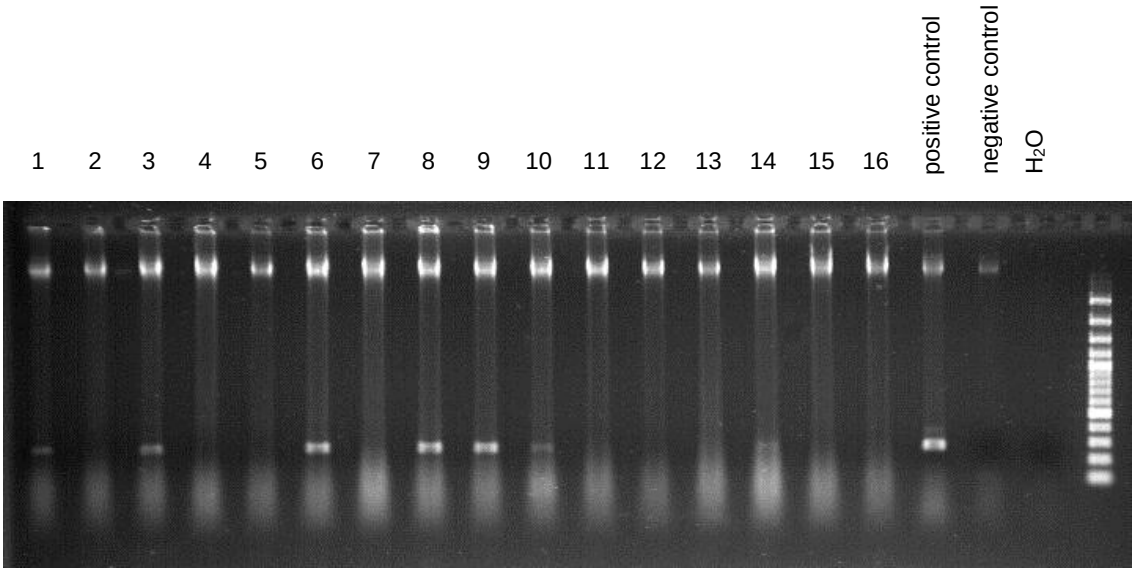

Figure S3. PCR analysis of wheat flower shows presence or absence of transgenes. Distinct white bands at the same height as the positive control indicate successful amplification of transgenic promoter regions (columns nr. 1, 3, 6, 8, 9, 10).

Table S1. Factors influencing the rate of cross-pollination of GM and non-GM wheat in experiment 1. This analysis of deviance table shows the effects of block and fertilizer (conditions under which parental plants grew in the 2008 field experiment), pollen recipient line identity (one contrast between Bobwhite GM and non-GM lines: "*Pm3b* vs. control"; two contrasts within GM lines: "*Pm3b*#2 vs. residual *Pm3b* lines" and "within residual *Pm3b* lines"; residual differences between control lines: "Control"), pollen donor line identity (one contrast between Frisal and Casana: "Frisal vs. Casana"; one contrasts between Frisal GM and non- GM lines: "Frisal GM vs. Frisal control"; one contrast between GM lines: "Frisal A9 vs. Frisal A13") as well as their interaction on the rate of cross-pollination. Abbreviations: df = degree of freedom, % DV = % deviance change due to addition of terms to model, F pr. = error probability based on approximate F-ratios (ratios of mean deviance changes).

Source of variation	df	% DV	F pr.
Block	3	3.1	0.052
Fertilizer application	1	0.2	0.461
Pm3b vs. control	1	16.1	< 0.001
Pm3b#2 vs. residual *Pm3b* lines	1	2.3	0.018
Within residual *Pm3b* lines	2	0.5	0.566
Control	3	1.2	0.400
Frisal vs. Casana	1	0.1	0.566
Frisal GM vs. Frisal control	1	0.0	0.924
Frisal A9 vs. Frisal A13	1	9.6	< 0.001
Pm3b vs. control × Frisal vs. Casana	1	0.0	0.935
Pm3b vs. control × Frisal GM vs. Frisal control	1	1.9	0.030
Pm3b vs. control × Frisal A9 vs. Frisal A13	1	0.2	0.444
Pm3b#2 vs. residual *Pm3b* × Frisal vs. Casana	1	0.3	0.352
Pm3b#2 vs. residual *Pm3b* × Frisal GM vs. Frisal control	1	0.7	0.183
Pm3b#2 vs. residual *Pm3b* × Frisal A9 vs. Frisal A13	1	0.0	0.992
Control × Frisal vs. Casana	3	3.5	0.035
Control × Frisal GM vs. Frisal control	3	1.3	0.365
Control × Frisal A9 vs. Frisal A13	3	0.0	1.000
Within residual × Frisal vs. Casana	2	1.1	0.260
Within residual *Pm3b* × Frisal GM vs. Frisal control	2	7.0	< 0.001
Within residual *Pm3b* × Frisal A9 vs. Frisal A13	2	7.6	< 0.001
Residual	110	43.3	
Total	145	100	

Table S2. Factors influencing cross-pollination rates in experiment 2. This analysis of deviance table shows the effect of the wind direction (west vs. east), distance to pollen source (divided into log(distance) and residuals), plant variety and line identity as well as their interactions on the rate of cross-pollination. Abbreviations: df = degree of freedom, % DV = % deviance change due to addition of terms to model, F pr. = error probability based on approximate F-ratios (ratios of mean deviance changes).

Source of variation	df	% DV	F pr.
Block	3	1.1	0.429
West vs. east	1	1.5	0.048
log(distance)	1	41.4	< 0.001
Residual distance	1	0.0	0.913
Bobwhite vs. Frisal	1	3.7	0.002
Pm3b#1 vs. *Pm3b*#2	1	0.6	0.224
West vs. east × log(distance)	1	0.5	0.281
West vs. east × residual distance	1	0.0	0.768
log(distance) × Bobwhite vs. Frisal	1	0.2	0.531
Residual distance × Bobwhite vs. Frisal	1	0.1	0.640
log(distance) × *Pm3b*#1 vs. *Pm3b*#2	1	0.9	0.127
Residual distance × *Pm3b*#1 vs. *Pm3b*#2	1	0.1	0.656
Residual	81	50.0	
Total	95	100.0	

GENERAL DISCUSSION

Discussing the results of the field experiments

Ecology of transgenic plants is an interesting and very broad topic. Genetically modified (GM) plants provide ecologists with a unique opportunity to study the effects of single genes on a plant's interactions with its environment — effects which are difficult to observe in natural plant populations where the individual genomes usually vary in many genes.

This thesis presents an insight into several important aspects of ecological performance of a common agricultural plant with introduced resistance genes to a fungal pathogen. Using the example of wheat, genetically modified with several different single genes conferring resistance to powdery mildew disease, we assessed performance of transgenic plants both in the glasshouse and under realistic field conditions in diverse biotic and abiotic environments. A special focus of the study was the response of transgenic plants to competition and their potential to persist and spread in agricultural and natural habitats, including the potential for the transgenes themselves to spread via seed or via pollen dispersal (cross-pollination). The availability of close genetic controls (null-segregants) for most of the GM lines used allowed a good comparison for many aspects of the plants' ecology, which has rarely been possible in previous ecological studies on transgenic plants.

The results obtained contribute not only to a better understanding of plant ecological questions in general but have also an applied value, in particular for environmental risk assessment of GM crops, for example providing recommendations for more optimal gene expression levels in transgenic plants and the potential use of transgenic genotype mixtures to reduce pathogen levels at stand level.

While individual chapters of this thesis are dealing with particular aspects and discuss the results of the experiments in a frame of a certain topic, here I try to summarize the results of all the experiments carried out over four years and to put them into a broader context of transgenic crop ecology.

Resistance of GM wheat lines to powdery mildew

Powdery mildew, caused by *Blumeria graminis* f. sp. *tritici*, is a common disease of wheat, widely distributed throughout the world. Powdery mildew causes major yield losses in wheat which can be as high as 45% (Fried *et al.* 1979), especially in humid regions and in years with mild temperatures and high humidity in spring (Bennett 1984, Lipps and Madden 1989). Nitrogen, which is almost always applied in wheat

cultivation, can even further increase the disease severity (Lipps and Madden 1989). Up to now, growing mildew resistant varieties is the most economical way to control powdery mildew. Wheat varieties often vary in their resistance to powdery mildew, which allows successful selection of resistant genotypes. Unfortunately, traditional selection is a long-lasting process whereas new mildew-resistant varieties usually have a limited period of usage because the pathogen tends to overcome the variety resistance with time and develops new races. Biotechnology offers a convenient tool to speed up obtaining resistant genotypes by "transplanting" the resistance genes from naturally resistant varieties or even other species, such as barley, to the high-yielding but susceptible wheat varieties. Additionally, biotechnology allows increasing gene expression many times compared to natural levels giving breeders an opportunity to potentially achieve complete resistance in a resulting transgenic plant.

In our experiments we used seven different lines of wheat with introduced transgenic resistance to powdery mildew pathogen. These lines were based on two genetic backgrounds: the genes were introduced to the genomes of the Mexican wheat variety Bobwhite and the Swiss wheat variety Frisal. Powdery mildew is not an important pathogen in Mexico with its low rainfall. The natural Bobwhite variety therefore does not have an effective defense against this pathogen and is highly susceptible to it. In contrast, the Swiss variety Frisal was previously used in agriculture and thus was resistant originally, but its cultivation was stopped in Switzerland in 2008 due to new pathogen races that could overcome the resistance.

The five transgenic lines based on the variety Bobwhite were modified with different alleles of the *Pm* resistance gene which was obtained from a resistant wheat cultivar Chul (Brunner *et al.* 2011). Our study showed that all the five transgenic Bobwhite lines (*Pm3b*#1, *Pm3b*#2, *Pm3b*#3, *Pm3b*#4 and *Pm3a*#1) were highly resistant to powdery mildew compared to their genetically close non-transgenic control lines. Although the severity of the infection and the differences between GM and control lines varied in different experiments depending on the biotic and abiotic environment, Bobwhite GM lines always showed lower infection levels than the corresponding control lines.

However, this was not the case for the transgenic lines based on the variety Frisal. The two transgenic Frisal lines A9 *Chi* and A13 *Chi/Glu* received the *chitinase* and *glucanase* genes from barley. These genes are related to non-specific broad resistance to fungal pathogens and were expected to increase resistance to powdery

mildew and possibly to some other wheat pathogens (Leah *et al.* 1991, Zhu *et al.* 1994). In our experiments, however, the natural variety Frisal itself was still quite resistant to the pathogen. Thus, all the Frisal lines, including GM and control, showed lower mildew infection rates than non-transgenic Bobwhite lines. Mildew infection in Frisal was also not more severe than in the modern cultivated wheat varieties Toronit, Fiorina and Casana, which were used as an additional control in our experiments. Only in the glasshouse, with high mildew pressure, and at an early stage of plant development we observed that transgenic Frisal lines were slightly more resistant than the non-transgenic mother variety Frisal (unpublished data).

I therefore conclude that the effect of the resistance transgenes can be easily observed in highly susceptible varieties such as Bobwhite but not in already resistant varieties such as Frisal. It is currently not clear if the genetic background was responsible for the lack of differences between transgenic and non-transgenic lines in Frisal or if the used transgenes *chitinase* and *glucanase* were not as effective as the *Pm* transgene. Further experiments testing all the different transgenes in each of the genetic backgrounds would be necessary to clarify this point.

Interestingly, the five transgenic lines based on Bobwhite showed differences among themselves in their resistance to powdery mildew. These differences could be linked to different levels of the transgene expression in these lines (Zeller *et al.* 2010, Brunner *et al.* 2011). Because the *Pm* gene was introduced under the control of an ubiquitin promoter from maize, the expression was enhanced more in some GM lines than in others compared to natural expression levels. This was the case, even though the same technique was used for genetic modification of Bobwhite to obtain each of the five transgenic *Pm* lines. This indicates that the place of the gene insertion into the genome, which could not be controlled, could have affected the level of expression. Line *Pm3b*#2 with the highest expression of the *Pm* transgene showed the lowest infestation with mildew. For the other four transgenic *Pm* lines there also was a correlation between expression level and resistance to powdery mildew. It would be interesting to test how the position of a transgene in the genome and the transgene expression level affect plant resistance, assessing more lines with different expression levels but the same gene of resistance.

Potential persistence and spread of transgenic wheat in the environment

One of the crucial questions and a serious public concern is the safety for the environment of growing a new transgenic crop. By genetic modification we often aim to introduce a trait which gives a plant an advantage over its non-transgenic relative. In the case of pathogen resistance, for example, the advantage could be a better growth and reproduction under disease pressure. However, GM plants with improved fitness can potentially also be more competitive than their conventional counterparts or could compete successfully with weeds and persist in natural plant communities. The chances for the plants carrying an "advantageous" gene to spread faster and possibly to become weedy and contaminating natural habitats or conventional seed lots can potentially be higher than for the non-transgenic crop (Claessen *et al.* 2005, Harker *et al.* 2005, Zapiola *et al.* 2008). Even if a transgenic plant does not pose an increased risk to spread compared to a conventional variety, small quantities of GM material among conventional crops can be intolerable due to legislative regulations regarding GM material, which are in power in many countries (Cellini *et al.* 2004, Ponti 2005, Messean *et al.* 2007, Das Schweizer Parlament 2012). It is therefore very important to know as much as possible about the ecological behaviour of transgenic plants in the field, their life-history traits and potential to grow, persist and reproduce in different habitats.

In a series of the experiments I assessed the persistence and growth of the transgenic wheat lines in the field with and without competition, among natural vegetation and in different crop stands; and under high and low soil nutrient levels (Chapters 1, 2, 3). We also evaluated the longevity of seeds in the soil seed bank under controlled conditions (Chapter 2) and natural outcrossing rates which define gene flow via pollen over short distances from GM to conventional wheat lines in the field (Chapter 6). The parameters assessed gave us a comprehensive understanding of the ecological behaviour of our studied plants under different environmental scenarios, in the glasshouse and under field conditions.

My assessment of the performance of transgenic wheat plants under different environmental conditions over several years showed that both GM and non-GM plants successfully grew and produced seeds both in pure stands and planted into wheat or weed communities. Despite carrying transgenes which led to high plant resistance to the fungal pathogen (Chapters 1 and 3), the transgenic lines did not perform better than their non-transgenic counterparts. These results did not provide evidence for improved

fitness of transgenic wheat plants neither in pure stands nor under competition in the crop or among weeds.

In fact, four tested GM lines based on Bobwhite had even lower performance than their control lines under competition, even when pathogens were present (Chapters 1 and 2); and the higher the resistance of the GM lines the lower was their competitive ability (see also the next section of this General Discussion). Under competition with the other wheat lines, weaker performance compared to control was also observed for the Frisal GM line A13 *Chi/Glu.* This line carried two transgenes coding chitinase and glucanase (Chapter 1). These low performances of transgenic lines could have been caused by physiological costs of resistance, which seem to become more evident under stressful conditions such as competition, which require higher resource investment (Bergelson and Purrington 1996, Dewitt *et al.* 1998, van Dam and Baldwin 2001, Cipollini 2002). The costs of resistance and their effect on plant fitness and competitive ability will be discussed in more detail in the next section.

A persistent seed bank in the soil can be one source for the potential spread of transgenic plants in the environment. How long seeds can persist in soil preserving their viability depends on environmental conditions such as soil temperature, moisture and oxygen availability, which in turn depend on soil type (Hanks and Thorp 1956, Dasberg and Mendel 1971, Lafond and Baker 1986). Overwintering of seeds or seedlings of transgenic wheat in the field could be a source of contamination for a subsequent non-GM crop. We therefore assessed seed viability of GM vs. non-GM wheat storing the seeds for up to 6 months in the climate chamber under conditions resembling temperature, oxygen and moisture conditions in the field in winter, and monitored persistence of the naturally occurring seedlings in the field throughout autumn, winter and in spring.

The field post-harvest monitoring showed that the seedlings of wheat which germinated in autumn were able to persist in the field over the whole winter. Although there were clear variety differences in over-winter mortality rates of the seedlings, I did not observe any differences in survival between the transgenic and conventional wheat lines. The Swiss wheat variety Frisal and the Frisal-derived transgenic lines, which should be more adapted to cold temperatures, expectedly showed higher survival rates than the non-transgenic and transgenic lines of the Mexican wheat variety Bobwhite. However, transgenic Bobwhite or Frisal lines did not differ in their persistence and mortality rates from the corresponding non-transgenic lines.

The results of the seed storage experiment showed that the seeds of wheat either geminate quickly or lose their viability within the first 3 months in soil, indicating that the soil seed bank is not an important source of possible transgene persistence and spread in wheat. This outcome of our study is in accordance with other published work on transgenic and conventional wheat cultivars reporting that a persistent soil seed bank is not common for wheat (Anderson and Soper 2003, Harker *et al.* 2005, Nielson *et al.* 2009). Successful overwintering of germinated seedlings in our experiments, however, indicates that volunteering GM seedlings can be a more important source of contamination of the subsequent conventional fields with transgenic wheat plants than the soil seed bank (Chapter 2).

Another source of transgene escape and spread which should be thoroughly assessed before the release of transgenic crops to the environment is gene flow via pollen (Andow and Zwahlen 2006, Mallory-Smith and Zapiola 2008). In field studies on GM crops, pollen flow over large distances is often assessed, because it gives an understanding of the potential of a transgene to escape outside the field; and such an assessment is necessary to recommend separation distances between conventional and GM fields (Luby and McNicol 1995, Snow 2002, Mallory-Smith and Zapiola 2008, Warwick *et al.* 2009). Knowledge about the outcrossing ability of the transgenic plant over short distances within the crop is, however, also important. The ability of the transgene to spread within the field or over short distance outside the field can potentially lead to the appearance of fit hybrids through outcrossing with conventional varieties or wild relatives or can cause undesired transgene stacking if several transgenic varieties are grown in close proximity. The potential of transgenic wheat to cross-pollinate would also determine if contamination of a seed lot or the field with minor quantities of transgenic seeds or volunteering plants could lead to further transgene spread among the conventional crop.

We carried out three experiments to assess the potential for cross-pollination over short distances within the field in transgenic vs. non-transgenic wheat (Rieben *et al.* 2011). Wheat is a predominantly self-pollinating species with outcrossing rates which reportedly do not exceed 1–2% (Vries 1971, Martin 1990, Gustafson *et al.* 2005). Relatively heavy pollen grains and the fact that self-pollination usually occurs before the florets even open lead to lower cross-pollination rates in wheat than it would be expected in many other wind-pollinated species (Vries 1971). Transgenic wheat is therefore presumed to pose low risks for pollen-mediated gene flow. This assumption

is, however, only based on the idea that transgenic plants would behave exactly the same as non-transgenic ones. In our study we were interested if the transgene might alter pollination biology of wheat and if outcrossing rates and the risk of transgene spread could be higher within the field over short distances in GM plants than in conventional wheat. A companion study investigated gene flow over larger distances outside the field (Foetzki *et al.* 2012).

Our findings indicate that the introduction of a new gene to a plant genome can in some cases unintentionally alter the plant outcrossing rates. Although the average outcrossing rates over short distances did not exceed 3.4% in our study, Bobwhite-based transgenic lines containing the *Pm3b* gene were overall six times more likely than non-GM control lines to produce outcrossed offspring (Chapter 6). Transgenic lines based on Frisal wheat variety also differed in their probability to be a pollen donor for cross-pollination, the line A9 *Chi* being more likely to pollinate the other genotypes in close proximity.

We also found that the outcrossing rate varied among four Bobwhite-based GM lines, some of which were much more likely to produce hybrid offspring with a cross-pollination rate as high as 8.5% (Chapter 6). Interestingly, two of the Bobwhite transgenic lines had altered spike morphology which could at least partially explain high outcrossing rates. The florets of such plants remained open for a prolonged period of time, possibly due to partial male sterility. Spread-open spikes and longer exposure of stigmata could create favorable conditions for pollination by pollen from external sources. However, one of the lines with altered spike morphology (*Pm3b#2*) did not show higher cross-pollination rates than the other GM lines. This inconsistency could have been due to generally very low seed production in this line, which showed signs of partial sterility (Chapters 3 and 6).

Summarizing the results of all the experiments about the potential of transgenic wheat to persist and spread in the environment, I conclude that the ability of transgenic wheat to persist and escape from the fields is case-dependent and should therefore be assessed for each new transgenic line separately. Overall, GM wheat lines with constitutive pathogen resistance did not survive better or persist longer than their conventional counterparts. The non-persistent seed bank in soil is also not a likely source of spread of transgenic wheat, at least in the countries with temperate climate and low winter temperatures. However, seedlings of transgenic wheat were able to survive winter and continue growth in spring. Furthermore, outcrossing rates were

higher for some GM lines than for non-GM wheat. The observed transgene side effects on plant pollination biology and strong variety differences in overwinter mortality rates of seedlings indicate that case-by-case assessment of new transgenic wheat lines for different stages of the plant life cycle and under a range of environments is necessary.

Costs of resistance impair performance of the plants with high transgene expression

Overexpression of the introduced gene is often the consequence or even the aim of genetic modification by the means of biotechnology. The genes are introduced with strong promoters which enhance gene expression in the host genome (Rooke *et al.* 2000, Yahiaoui *et al.* 2004). This allows achieving much higher levels of the gene expression in transgenic plants than it would be observed in nature or in a conventional crop.

The different transgenic lines used in our experiments had up to several-hundred-fold increased expression levels compared to the mother varieties from which the genes were obtained (Yahiaoui *et al.* 2004, Brunner *et al.* 2011). In the GM lines based on the Bobwhite wheat variety, this led to a strongly increased resistance to powdery mildew fungal pathogen compared to the conventional Bobwhite and control lines (Chapters 1–4). Despite the decrease in the pathogen incidence, the yield, however, did not improve in these lines compared to control lines, even under pathogen pressure.

Instead, we observed fitness reductions in *Pm3b* transgenic lines compared with corresponding non-transgenic control lines, this reduction being higher in the GM lines with higher expression of the transgene. The line *Pm3b*#2, which according to the expression analysis had several times higher transgene expression levels than the other Bobwhite-based transgenic lines (Zeller *et al.* 2010, Brunner *et al.* 2011), showed the highest yield reductions among all GM lines and was most sensitive to competition (Chapters 1–3 and 5). In contrast, another Bobwhite-based transgenic line, *Pm3b*#3, which showed segregation in transgene expression and a high proportion of plants with low transgene expression, had only minor or no side effects on fitness and developed the highest yield among all the Bobwhite-based GM lines (Chapters 1 and 3). About 44% of the plants of this line showed different degrees of susceptibility to powdery mildew in the sixth generation leading to a higher average mildew infection compared to the other three *Pm3b* transgenic lines (Brunner *et al.* 2011).

The differences in gene expression and the consequent differences in performance between the four *Pm3b* transgenic lines based on the same genetic background could be an effect of the transgene position in the genome known to be able to influence gene transcription (Stam *et al.* 1998, Stoger *et al.* 1999, Rooke *et al.* 2000). Each of the *Pm3b* transgenic lines used in our experiments was created by a separate insertion event. The insertion position of the gene in the genome was, however, random and could influence the transgene expression level (Chapter 3).

Interestingly, apart from overall lower yield, we also observed a more sensitive response to competition and to other stresses in some transgenic lines. For example, Bobwhite transgenic lines responded more sensitively than the corresponding control lines to spraying fungicide in the glasshouse, showing reduced fitness and chlorotic leaves (Chapter 3) and to competition from neighboring plants in pure stands, in other wheat backgrounds (Chapter 1) and in weed communities (Chapter 2). Such fitness differences between Bobwhite GM and control wheat lines were not observed or were less pronounced when the plants were grown on their own with greater distances between plants, i.e. without competition (Chapter 2). This finding is in line with the published studies on conventional plants where weaker relative performance has been shown for resistant plants subjected to competition or other environmental stress such as, for example, nutrient limitation (Heil *et al.* 2000, van Dam and Baldwin 2001).

These results support the view that constitutive expression of resistance transgenes can withdraw available resources from other important processes and thus incur higher physiological costs for the resistant plant, resulting in fitness costs (Dewitt *et al.* 1998, Tollrian and Harvell 1999). Constant allocation of resources to transgenic defence becomes especially "costly" for the plant when other resource-intensive responses are induced by the environment. Our data also show that the level of the transgene expression might play an important role affecting the size of resistance costs — an important effect to consider in the discussion of pros and cons of stacking multiple transgenes in one genotype vs. growing mixtures of transgenic plants (Zeller *et al.* 2012).

Large transgene × environment interactions call for GM trials under realistic field conditions

Although the fact that plants sometimes perform very differently in the glasshouse than in the field is often referred to as "common knowledge" by plant breeders, to our

knowledge, no studies have been published so far that show that exposing a transgenic plant to field conditions can trigger large changes in performance compared to the controlled environment in the glasshouse, especially when similar changes are not observed in non-transgenic sister lines. Thus, there is even a point of view that, if a GM plant differs from the control in the expression of one gene in the laboratory, testing such a plant in the field may not be necessary — the principle of substantial equivalence (Raybould 2006, 2010). These ideas were incorporated into some legal documents and are currently used in the official guidelines for risk assessment of transgenic crops (OECD 1993, FAO 2009). Rather contradictory to this concept, our results showed that the environmental conditions in the field can affect and even reverse the performance differences between GM and control plants observed under standardized conditions in the glasshouse (Chapter 3).

Many phenotypic effects and differences in yield between our GM and control wheat lines were not detectable in the glasshouse but became evident under realistic field conditions. Transgenic *Pm3b* lines, for example, showed better performance and higher yield compared to non-GM control lines under glasshouse conditions with very high mildew infection levels but no other environmental stresses such as drought or competition. In the field, however, the performance of these lines was impaired in comparison to the non-GM control. The most probable reason for this performance reversal was higher level of environmental stress in the field. Under field conditions during the vegetation season, the plants were subjected to periodic water limitations, competition from neighbors, infestations by other fungal pathogens and herbivore attack. Along with constant expression of the resistance transgene to powdery mildew, the response to other stresses seemed to exhaust available resources in GM plants, resulting in their poor performance in the field compared to non-transgenic wheat plants. These differences could not be seen in the glasshouse experiment where stable controlled conditions were maintained, competition and other pathogens were absent and plants were regularly watered.

Stronger competition or stress made the difference GM vs. control more evident. This was, for example, observed in the field experiments with different competitors, or in the glasshouse when part of the plants was sprayed with a high dose of fungicide and *Pm3b* lines with the highest resistance to powdery mildew showed a more sensitive response (leaf chlorosis and overall weaker performance). Similar effects were previously found in conventional plants with induced resistance to

pathogens or herbivores: under competition the plants with induced defence performed less well than non-resistant plants (Agrawal 2000, van Dam and Baldwin 2001). The limited resources thus cannot be used simultaneously and equally effectively for several defensive or stress responses in plants without negative trade-off effects on plant fitness (Bergelson and Purrington 1996, Dewitt *et al.* 1998, Heil *et al.* 2000, Heil 2001, Cipollini 2002, Strauss *et al.* 2002).

Interestingly, not only fitness differences between GM and control lines were found in the field but not in the glasshouse. Some unintended phenotypic effects also became evident only under field conditions. Across our experiments, we observed the following unintended phenotypic effects: chlorotic leaves, increased ergot infection and changed spike morphology in lines *Pm3b*#2 and *Pm3b*#4. Unintended phenotypes appeared more often in lines with higher resistance to mildew (lower mildew incidence). In particular, line *Pm3b*#2 which had the highest gene expression and the lowest mildew incidence among all the GM lines showed not only the lowest yield among all the *Pm3b* lines (56% reduction in yield in 2008) but also most of the unintended phenotypes such as chlorotic leaves, changed spike morphology and high infestation with ergot pathogen (Chapter 3). These effects were only found in highly resistant plants in the field but not in the glasshouse.

Strong genotype × environment interactions observed in transgenic wheat point out the importance of the field tests with transgenic plants under a range of different environments which would allow uncovering potential changes in plant ecological behaviour in case such changes happened after transgene introduction.

Using mixtures of GM lines as an alternative to gene stacking to increase crop resistance and yield

Numerous ecological and agricultural studies have shown that more diverse plant communities are more productive and can also provide other benefits such as improved community functions, better resource use, increased resistance to pathogens, prevention of pathogen outbreaks and slowing evolution of counter-resistance in pathogens (Schmid 1994, Tilman *et al.* 1996, Hector *et al.* 1999, Rausher 2001, Mundt 2002, Roscher *et al.* 2005, Balvanera *et al.* 2006, Schmid *et al.* 2008, Haddad *et al.* 2011). In particular, it has been shown, that cultivar and multiline crop mixtures can be especially beneficial for preventing powdery mildew and other fungal disease spread in grain crops (Mundt 2002). Higher genetic diversity of the crop stand could also contribute to

achieving sustainability in agriculture, better yield stability, increased biodiversity and visual diversification of agricultural landscapes (Wolfe 1985, Smithson and Lenne 1996, Wolfe 2000).

Despite the benefits, practical use of mixed crop stands in agriculture is, however, not popular among agricultural producers and farm estates. Growing crop mixtures is currently limited by costs of separate harvest or post-harvest separation of non-homogenous crop stands and legal requirements for uniformity of seed material and varieties (Smithson and Lenne 1996).

We experimented with the mixtures of different transgenic and conventional lines of wheat assessing yield and powdery mildew infestation rates in monocultures and mixed stands with different GM-richness and GM-concentration, both at a plot and individual plant level (Chapter 4). We found that higher diversity of the genotypes and a higher proportion of GM lines in mixture leads to higher resistance to the pathogen and better yield than it would be expected from the average performance of corresponding monocultures. These results are in accordance with the ecological theory that better resource use or pathogen defence in a more diverse plant community can improve overall community performance (Tilman *et al.* 1996, Hector *et al.* 1999, Roscher *et al.* 2005, Cardinale *et al.* 2011).

The GM lines having the same single gene of resistance introduced but in different locations in the genome or carrying different alleles of the same gene showed an effect of transgressive overyielding and higher resistance to powdery mildew in the mixtures. Powdery mildew resistance increased with GM-concentration and with GM-richness, i.e. growing different GM lines together led to lower disease rates and better yield. Mixtures of a GM line with a control line were also less infected with powdery mildew than expected from the means of the two monocultures, supporting the view that if in a plant community a certain proportion of individual plants are resistant against a specific pathogen they can reduce the spread of infection (Browning and Frey 1969, Schmid 1994, Wolfe 2000).

While cultivation of species or variety mixtures might need additional efforts on developing new harvesting or post harvest segregation techniques, transgenic lines with the same genetic background and similar morphology but differing in resistance genes could provide an alternative and allow growing line mixtures with increased pathogen resistance, improved performance and a homogenous crop stand.

A modern development in biotechnology is stacking several genes together (gene pyramiding) thus tackling more complex physiological pathways or combining several types of resistance in a single plant genome (Datta *et al.* 2002, Servin *et al.* 2004, Bravo and Soberón 2008). Pyramiding transgenes could allow combining different defenses in one plant, achieving better and durable resistance against pathogens and possibly delaying the pathogen to host resistance evolution (Liu *et al.* 2000, Zhao *et al.* 2003). However, it remains unclear how simultaneous expression of multiple defense responses affects plant performance and yield. Ecological theory suggests that high costs of resistance may occur which could affect plant fitness (Bergelson and Purrington 1996, Dewitt *et al.* 1998, Purrington 2000, Heil 2001, Brown 2002, Heil and Baldwin 2002, Strauss *et al.* 2002). In addition, there are also concerns that stacking several resistance genes in one plant could lead to the evolution of "super-pests" that overcome such multiple resistance (Zhao *et al.* 2003). Growing multiline mixtures of transgenic plants which differ in resistance traits might be a better alternative allowing higher resistance and yield but avoiding high physiological costs of resistance and creating lower selection pressure on the pests than monoculture of uniform lines with multiple resistances.

Ecology of GM crops: conclusions and recommendations

Single genes are acting within a complex plant genome in close relation with the other genes and physiological mechanisms, their transcription being affected by numerous internal and external factors (Stam *et al.* 1998, Somerville and Somerville 1999, Stoger *et al.* 1999, Rooke *et al.* 2000). Considering the effects of single genes introduced by the means of biotechnology, this complexity should be taken into account: introducing a trait without affecting other plant characteristics is still a challenge and unintended side effects are commonly found in transgenic plants (Cellini *et al.* 2004, Filipecki and Malepszy 2006, Zeller *et al.* 2010). Many of these side effects are not fitness- or safety-related; many can be detected early or can be overcome by subsequent plant breeding. However, the very presence of unintended effects in transgenic plants and the fact that these effects can sometimes be only observed under certain environmental conditions (Cellini *et al.* 2004, Filipecki and Malepszy 2006, Zeller *et al.* 2010) indicate that the issue of genetic modification is much more complex than transplanting a single gene (and a single trait) from a non-related species. Effects on other plant traits or plant ecological behaviour are likely to be observed, and extensive field tests and

conventional breeding procedures are usually needed before newly produced transgenic plants become suitable and approved for agricultural use.

The same principle of complexity applies to the ecology of transgenic plants. GM plant performance and ecological behaviour can only be considered in a context of a complex set of plant interactions with biotic and abiotic environmental factors and can only be assessed under a range of realistic environmental conditions.

Strong transgene × environment interactions observed in transgenic wheat (Chapter 3) underline the importance of testing transgenic plants under field conditions in which these plants are likely to be cultivated in the future. As our study showed, moving experiments from the laboratory to the field can reveal differences between transgenic and conventional plants not observed under stable controlled conditions and can even reverse the difference in performance of GM vs. control plants. Moreover, some unintended effects could be only discovered under field conditions (Zeller *et al.* 2010). While the principle of substantial equivalence (OECD 1993, Raybould 2006, 2010) used in some guidelines for risk assessment of transgenic crops may be useful to compare plant metabolic profiles under controlled conditions in the laboratory, such testing cannot replace field trials under a set of diverse environmental conditions. Plant performance can change dramatically when a plant is subject to realistic field conditions. GM plants which showed no differences from their non-GM controls under optimal conditions in the lab differed significantly in important fitness-related traits when exposed to competition in a crop stand or to other environmental stresses, i.e. to the conditions which will inevitably occur during their future cultivation.

In reality, the GM plant may occur not only in its own monoculture stand but also in other agricultural or natural habitats — the assumption lying at the base of requirements for GM-crop risk assessment (Linder and Schmitt 1994, Conner *et al.* 2003, Andow and Zwahlen 2006). Plant competitive ability, plant fitness and persistence at different stages of the life cycle would play especially important role in such "foreign" environments, affecting a plant's ability to reproduce and spread its offspring among non-GM crops or wild plants (Allard and Adams 1969, Fredshavn *et al.* 1991, Kalinina *et al.* 2011). The GM wheat lines used in our study did not show improved fitness or competitiveness under the field conditions. Four of the GM lines, in fact, showed weaker performance than their non-GM relatives due to the costs of resistance observed in the field. Two of the GM lines showed multiple unintended effects and altered cross-pollination rates when subject to competition or other

environmental stresses in the field (Chapters 1–3). The same GM lines, however, showed only small or no difference from control lines being planted without competition (Chapter 2) or even benefited compared to control lines under stable and more favorable conditions in the glasshouse (Chapter 3). Contrary to the ideas of simplifying pre-production risk assessment tests (Raybould 2010), simulating the wide range of environments in which transgenic plants are likely to occur is important and will improve the quality and reliability of environmental risk assessment of transgenic crops.

The costs of resistance observed in transgenic wheat lines with high resistance to powdery mildew led to lower yields compared with non-GM control lines, thus lowering the potential value of such lines for agriculture (Chapters 1–3, 6). The lines with the highest resistance to powdery mildew showed the highest yield penalties. This finding supports ecological theory about allocation costs and limits to plant plasticity (Bergelson and Purrington 1996, Dewitt *et al.* 1998, Heil 2001, Brown 2002, Heil and Baldwin 2002) and questions the benefits of introducing strong constitutive resistance and high transgene overexpression in GM plants. Currently the transgenes are introduced with strong promoters which often increase their natural levels of expression many-fold (Christensen and Quail 1996, Rooke *et al.* 2000). The constant expression of the transgene conferring constitutive resistance to the plant also means constant resource investment to unnecessary (in the absence of a pathogen) defence, while producing the gene product in larger quantities than it would happen in nature incurs even higher resource allocation to these processes. From an ecological point of view, it would be more advantageous to introduce inducible pathogen resistance to transgenic plants or to develop more advanced promoters which would allow transgene expression localized to the plant tissues where defence is most needed and most effective (Brunner *et al.* 2011).

Overexpression of the transgene can ensure high or even complete resistance to the pathogen. Despite these highly resistant plants may seem to be attractive for agricultural production lowering costs for fungicide use, they might not be sustainable at a large scale due to the costs of such a "resource-intensive" resistance, reducing the yields. A balance between resistance, transgene expression level and tolerable costs of resistance has to be found to ensure stable high yields and pathogen resistance at lowest possible physiological cost. Transgene overexpression may be an effective way to achieve high resistance in plants but it might not be an optimal method to raise the

yields due to resource allocation trade-offs, well-known from ecological studies (Bergelson and Purrington 1996, Heil *et al.* 2000, Brown 2002, Strauss *et al.* 2002). I suggest using transgenic lines with lower rates of transgene expression which could still have effective resistance to the pathogen but at lower costs, as it was observed in *Pm3b*#1 and *Pm3b*#3 GM lines in our study.

It is conceivable that the extent of the costs of resistance is usually environment-dependent (van Dam and Baldwin 2001, Cipollini 2002, Strauss *et al.* 2002, Siemens *et al.* 2003). In our study we observed higher costs in GM plants subject to competition, while the costs were low without competition in the field or even absent in the glasshouse experiment (Chapters 1–3). The costs of resistance increase when the plant is subject to additional environmental stress, requiring another response and thus additional resource investment (Dewitt *et al.* 1998, Heil 2001, van Dam and Baldwin 2001, Cipollini 2002, Strauss *et al.* 2002).

A modern direction in biotechnology is stacking, or pyramiding transgenes of resistance, which aims to combine multiple genes responsible for different plant defence mechanisms in one genome and to achieve durable broad-spectrum resistance (Datta *et al.* 2002, Servin *et al.* 2004). Based on ecological theory, however, it can be expected that simultaneous expression of several genes responsible for plant defences in a single plant genome will result in high physiological costs for the plant causing negative yield-defence trade-offs (Bergelson and Purrington 1996, Brown 2002). Using mixtures of transgenic lines with different resistances could be a wise alternative. As our study and the results of previous experiments on natural and agricultural species have shown, growing multiline crop stands can be beneficial, increasing yields, preventing pathogen spread and challenging fast pathogen co-evolution (Wolfe 1985, Smithson and Lenne 1996, Mundt 2002, Zeller *et al.* 2012). Moreover, because of better resource use by a more diverse plant community (Hector *et al.* 1999, Balvanera *et al.* 2006), mixtures could be advantageous on a longer prospective, providing more food security in regions with unstable climatic conditions. Biotechnology could bring a new dimension to the discussion of pros and cons of growing multiline mixtures offering a solution to one of the limitations of mixture use in agriculture — morphological heterogeneity of the crop stand and thus technical difficulties at harvest. Morphologically similar lines based on the same or similar genetic background but carrying different advantageous transgenes could provide similar benefits as transgene

stacking, at the same time minimizing negative trade-offs which are likely to occur between multiple "physiologically costly" resistance and productivity.

Biotechnology is undoubtedly an effective tool to speed up the first stages of a breeding process, to create additional genetic variability in a crop by introducing the genes which cannot be transferred or combined in one genome by means of traditional plant breeding. It opens new horizons for agricultural production, allowing, for example, to enhance crop resistance to limiting environmental factors (Bohnert and Jensen 1996, Wang *et al.* 2003) or to improve nutritional quality of food and food security in developing countries (Bouis 2007, Qaim 2010). Biotechnology cannot, however, completely replace the functions of a common plant breeding and does not deliver new crop varieties directly to the market. New transgenic plants have to go through thorough field tests and environmental risk assessment on a case-by-case basis, and usually must undergo additional breeding processes before they can be finally released for open-field cultivation.

As my study revealed, some genetic modifications of plant genomes expected to improve yield may interfere with the other plant processes leading rather to an opposite effect under certain environmental conditions. More effective use of currently available ecological knowledge and ecological methods could help to reveal or avoid unintended effects and ecological trade-offs in transgenic plants. Ecological knowledge about plant and community functioning could also offer alternative or complementary solutions to biotechnology to increase yields and achieve higher sustainability in modern agriculture. Some examples of such solutions are diversification of crop stands (Smithson and Lenne 1996, Mundt 2002), new breeding strategies based on ecological theory and aimed to improve group vs. individual fitness (Weiner *et al.* 2010) and weed suppression, increasing yields in crop stands with altered sowing patterns and density (Olsen *et al.* 2005). Biotechnology has therefore to be combined with the methods of conventional plant breeding, current knowledge about crop ecology and ecological theory in order to achieve the challenging goals of contemporary agriculture towards higher sustainability of crop production and effectively addressing the increasing world demand for food.

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SUMMARY

Harvesting field experiments by hand at ART Reckenholz in 2010

Modern agriculture faces challenges to supply food for a still growing world population and to improve sustainability of agricultural production. Spread of fungal diseases annually results in high yield losses of major cereal crops and demands high chemical input to agricultural fields. Although disease-resistant cereal varieties can be developed by the means of classical breeding, this process is time-consuming and resistant varieties have to be replaced after some time of cultivation due to pathogen co-evolution and developing counter-resistance. Biotechnology could speed up the breeding process, introduce new genes of resistance from other species and also contribute to sustainability in agriculture by reducing the use of chemicals and improving plant productivity under adverse environmental conditions. There are, however, concerns that transgenic plants may behave differently from their conventional relatives, contaminate conventional varieties through seed or pollen dispersal, spread and persist in- and outside crop fields or even become super-weeds due to their high resistance to pathogens or pesticides. To date little is known about the ecology of transgenic plants with introduced pathogen resistances. I carried out several glasshouse and field experiments to compare performance, competitiveness, persistence and ability to spread of seven pathogen-resistant transgenic wheat lines and conventional wheat lines and varieties in different biotic and abiotic environments.

The results showed that five transgenic lines based on the Mexican wheat variety Bobwhite had higher resistance to the fungal pathogen powdery mildew compared to their conventional control lines, whereas no improvement was observed in resistance of two transgenic lines carrying *chitinase* or *chitinase* and *glucanase* transgenes and based on the already resistant wheat variety Frisal. Despite high resistance, most of the transgenic lines did not deliver higher yield than their corresponding non-transgenic control lines. Most of the GM lines showed some deficiencies in fitness-related traits, this effect being especially pronounced in transgenic lines with strong overexpression of the transgene or with two transgenes of resistance and under environmental stresses, such as competition or fungicide spraying. The impaired fitness of the transgenic lines could be explained by the costs of resistance which occur due to the allocation of resources to constitutive defence against pathogens. The costs become stronger when the plant has to respond simultaneously to the pathogen attack and to other environmental stresses. Apart from impaired fitness and competitiveness, some transgenic lines showed unintended phenotypic effects, for example leaf chlorosis, changed spike morphology or hyper-sensitivity to fungicide

spraying. The costs of resistance and many unintended effects were most pronounced in transgenic lines with strong transgene overexpression and highest resistance. I conclude that very high expression of the transgene is not beneficial for plant performance because of high physiological costs. A balance should be found between the minimum level of expression sufficient to maintain resistance and reasonable costs which would allow increased yield.

My results also indicate that transgenic plants can respond to the environment differently than expected from genetically similar non-GM plants. Transgenic wheat lines showed higher decreases in fitness than control lines when grown with other wheat lines or among weeds. We observed strong performance differences in GM *versus* control lines between glasshouse and field experiments: *Pm3b* transgenic lines benefited in yield in the glasshouse but showed lower yield and unintended effects under the open-field conditions. Due to costs of resistance, low competitive ability and short-lived seeds in soil, the transgenic lines used in this study are not likely to spread and persist more effectively than their conventional counterparts. Transgenic plants, however, were able to persist throughout winter in the field and showed higher outcrossing rates over short distances than did control lines, indicating that their pollination biology was affected. Thus, persistence of seedlings and pollen flow within the field could be a source of contamination of the subsequent conventional fields with transgenic wheat. Mixtures of transgenic lines or transgenic and conventional lines showed higher yields and resistance to powdery mildew than monocultures. Growing mixtures of GM lines could be an alternative to staking multiple genes in single genome, which could cause high physiological costs due to multiple gene expression in a single plant.

We conclude that it is still challenging for biotechnology to produce transgenic plants without unintended effects affecting other plant traits and interactions with the environment. Strong transgene × environment interactions and line differences observed in this study underline the importance to assess the performance and ecological behaviour of transgenic plants under a broad spectrum of environmental conditions in the field, on a case-by-case basis. Although biotechnology can be a useful tool to address the problems of modern agriculture extending crop genetic diversity and shortening breeding processes, ecological knowledge and further development of conventional farming approaches, such as diversification of crop stands, also hold the potential to increase agricultural production and contribute to agricultural sustainability.

ZUSAMMENFASSUNG

Wheat plots covered with bird-protection net at ART Reckenholz in 2010

Um sowohl der steigenden Nachfrage nach Nahrungsmitteln als auch den globalen Umweltzielen gerecht zu werden, muss die moderne Landwirtschaft muss nachhaltiger werden und gleichzeitig die für höhere Erträge sorgen. Bei den wichtigsten Nutzpflanzen führen jedoch Pilzkrankheiten zu Ernteverlusten und hohem Pestizideinsatz. Krankheitsresistente Sorten können durch klassische Pflanzenzüchtung geschaffen werden. Diese Methode benötigt jedoch viel Zeit und resistente Sorten müssen ständig ausgewechselt werden, weil sich Schädlinge Resistenzen entwickeln. Die Grüne Gentechnik könnte mithilfe von Transgenen diesen Züchtungsprozess beschleunigen und Pflanzen schaffen, die mit geringerem Pestizideinsatz auskommen oder auch bei schlechten Umweltbedingungen gute Erträge liefern. Es gibt jedoch Bedenken dass sich transgene Pflanzen anders als konventionelle Pflanzen verhalten, durch Fremdbestäubung zur Kontamination fremder Felder und sich aufgrund ihrer Resistenzgene als „Super Unkräuter“ unkontrolliert in der Landwirtschaft und Natur ausbreiten könnten. Es gibt relativ wenige Studien, welche die Ökologie von gentechnisch veränderten Pflanzen untersuchen. Wir haben Gewächshaus- und Feldexperimente mit sieben krankheitsresistenten, transgenen Weizenlinien durchgeführt um zu erforschen, wie agronomische Messzahlen, die biologische Fitness sowie die Fähigkeit zur Persistenz und Ausbreitung durch biotische und abiotische Umweltfaktoren beeinflusst werden kann.

Fünf transgene Weizenlinien, basierend auf der mexikanischen Sorte Bobwhite, waren resistenter gegen die Pilzkrankheit Mehltau als nicht gentechnisch veränderte Kontrollinien. Im Gegensatz dazu zeigten zwei auf der Sorte Frisal basierende Linien (*Chitinase, Chitinase* und *Glukanase*) keine verbesserte Pilzresistenz. Die meisten gentechnisch veränderten Linien hatten jedoch eine tiefere biologisch Fitness als die nicht veränderten Kontrollinien sowie weitere unerwünschte Merkmale. Diese negativen Effekte waren bei Linien mit starker Transgen-Überexpression oder Linien mit zwei verschiedenen Transgenen am stärksten. Zudem wirkte sich Stress in Form von Konkurrenz mit Nachbarpflanzen oder Fungizidbehandlungen besonders negativ auf diese Linien aus. Die reduzierte Fitness der transgenen Linien kann durch die hohen Resistenzkosten erklärt werden. Diese entstehen möglicherweise weil die Resistenzgene auch bei Abwesenheit der Schadpilze exprimiert werden. Neben der geringeren Fitness und Kompetitivität machten sich bei einigen der transgenen Linien unerwünschten phänotypischen Merkmale wie Blattchlorose, veränderte Ährenmorphologie und Fungizid-Hypersensitivität bemerkbar. Aufgrund dieser

Resultate schliessen wir keine gentechnisch veränderte Pflanzen mit starker Transgen-Überexpression hergestellt werden sollten. Die Expressionsstärke sollte so eingestellt werden, dass die Vorteile durch die Pilzresistenz in jedem Fall grösser als die Resistenzkosten sind.

Unsere Resultate weisen zudem darauf hin, dass transgene Pflanzen manchmal anders auf Umwelteinflüsse reagieren als nicht veränderte Kontrollinien. Im Vergleich zu diesen erlitten transgene Pflanzen stärkere Fitnesseinbussen wenn sie in Konkurrenz zu anderen Weizensorten oder Unkräutern standen. Es kam auch drauf an, wo diese Experimente durchgeführt wurden: Linien mit *Pm3b* Resistenzgenen erzielten im Gewächhaus grössere Erträge als Kontrollinien; im Feld waren die Erträge aber tiefer und unerwünschte phänotypische Merkmale traten auf. Gerade wegen der hohen Resistenzkosten und der daraus resultierenden geringen Fitness dürfte das Risiko von unerwünschter Persistenz oder Ausbreitung der gentechnisch veränderten Linien geringer sein als bei konventionellen Sorten. Die Transgene Linien waren aber durchaus in der Lage den Winter im Feld zu überleben und zeigten zudem über kurze Distanzen höhere Kreuzungsraten als die Kontrolllinien. Dies wohl Aufgrund veränderter Bestäubungsorgane. Es ist deshalb möglich, dass sich die überlebenden Keimlinge im Jahr nach dem Anbau im selben Feld mit konventionellen Sorten vermischen. Mischungen von gentechnisch veränderten Linien erzielten, verglichen mit Monokulturen, bessere Pilzresistenz und höhere Erträge. Mischungen von gentechnisch veränderten Linien könnten deshalb als Alternative zu Pflanzen mit mehreren Transgenen genutzt werden dadurch die Resistenzkosten verringert werden können.

Wir stellen fest, dass es für die Biotechnologie immer noch schwierig ist transgene Pflanzen ohne unerwünschte Merkmale herzustellen. Die aufgetretenen starken Interaktionen zwischen dem Transgen und der Umwelt sowie Unterschiede zwischen den untersuchten Linien deuten darauf hin, dass transgene Linien verschiedenen Umwelteinflüssen ausgesetzt und jeweils einzeln (case-by-case) untersucht werden müssen. Die Biotechnologie kann möglicherweise einige Probleme in der Pflanzenzüchtung verringern und die Herstellung von neuen Sorten beschleunigen. Wenn richtig angewandt könnte jedoch auch unser ökologisches Wissen zu Verbesserungen in der konventionellen Anbaupraxis führen; beispielsweise indem resistente Sortenmischungen angepflanzt werden. Um die Landwirtschaft produktiver und nachthaltiger zu machen müssen verschiedene Strategien parallel verfolgt werden.

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Harvest of the field experiments in 2010

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Publications

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Reviewing

Reviewer for the following scientific journals:

- Journal of Applied Ecology
- Journal of Transgenic Research
- PLoS ONE
- Journal of Agricultural Science and Technology